공학박사학위논문

Development of a modified harvest system for the enhancement of recombinant human Factor VIII yield in alternative tangential flow perfusion culture

Alternative tangential flow perfusion 배양에서 재조합 인간 혈액응고 제8인자 생산성 향상을 위한 개선된 회수장치 개발

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Development of a modified harvest system for the enhancement of recombinant human Factor VIII yield in alternative tangential flow perfusion culture

A Thesis

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By

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ABSTRACT

The purpose of this thesis is to provide an alternating harvest method for enhancing Factor VIII (FVIII) yield in alternating tangential flow (ATF) perfusion culture. This goal was accomplished by developing a modified harvest system composed of a check valve, a pinch valve, and a timer.

This thesis was composed of three studies. In the first study, three cell retention devices, an ATF system, a spin-filter, and a Centritech Lab III centrifuge was evaluated for the production of recombinant human Factor VIII (rhFVIII) co-expressed with von Willebrand factor (vWF). From the results, it was found that the FVIII activity (FVIII:C) in bioreactor was significantly higher in the ATF perfusion culture than two other perfusion cultures. Moreover, the FVIII:C recovery yield was unexpectedly low in the ATF perfusion culture. Therefore, the reasons for this low FVIII:C recovery yield were studied. It was revealed that the inactivation and the surface adsorption of FVIII onto the harvest bag were not the main reasons for the low yield in the ATF perfusion culture. The FVIII:C recovery yield was not increased by the use of a hollow fiber filter with 0.5 μm pore size instead of 0.2 μm pore size. Additionally, the retention of FVIII molecules by the hollow fiber filter was a dominant factor in the low FVIII:C recovery yield in the ATF perfusion culture. It was demonstrated that FVIII yield was significantly improved by controlling transmembrane pressure (TMP) across the hollow fiber filter membrane. Taken together, these results suggest that TMP control could be an efficient method for the enhancement of FVIII yield in an ATF perfusion culture.

In the second study, it was described the development of a modified harvest system to enhance FVIII yield in ATF perfusion culture. The main innovation of the modified harvest system is the use of check and pinch valves, eliminating the need of a peristaltic pump for

harvest.

In the third study, the modified harvest system was applied to perfusion cultures of Chinese hamster ovary (CHO) cells, which co-express both rhFVIII and vWF. The modified harvest system showed comparable cell growth with the conventional harvest system using a peristaltic pump. The perfusion rate was successfully controlled using the system. In addition, the modified harvest system achieved an approximately 13.6-fold increase in the final recovery yield of FVIII:C and a 1.47-fold increase in the product yield of FVIII:C compared with a peristaltic pump. Enhancement of the yield of FVIII:C resulted from the reduction of FVIII antigen (FVIII:Ag) retention. As a result of TMP measurement, the reduction of the retained FVIII:Ag was due to the increased TMP, which was caused by the characteristic function of a check valve, compared with a peristaltic harvest system.

In conclusion, the modified harvest system developed in this thesis could be useful to enhance the product yield of other recombinant proteins in ATF perfusion culture. Another important point is that the system provides a promising tool to solve the fouling problem in the filtration-based perfusion system, especially in ATF perfusion culture.

Key words: Factor VIII, ATF perfusion, Modified harvest system, Transmembrane pressure, Pinch valve, Check valve

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

FVIII	Factor VIII
FVIII:Ag	Factor VIII antigen
FVIII:C	Factor VIII activity
vWF	von Willebrand Factor
vWF:Ag	von Willebrand Factor antigen
ATF	Alternating tangential flow
CHO	Chinese hamster ovary
TMP	Transmembrane pressure
DO	Dissolved oxygen
ELISA	Enzyme linked immunosorbent assay
rFVIII	Recombinant Factor VIII
BDD-rhFVIII	B-domain deleted recombinant human Factor VIII
vWD	von Willebrand disease
DHFR	Dihydrofolate reductase
P_{in}	Inlet pressure
P_{out}	Outlet pressure
P_{perm}	Permeate pressure

Chapter I

Introduction

1.1 Factor VIII

Factor VIII (FVIII) is an X-linked gene product that accelerates the activation of Factor X by Factor IX in the coagulation process. Human FVIII is an essential cofactor in the blood coagulation cascade. A deficiency in the amount of FVIII in the blood results in hemophilia A which is an inherited bleeding disorder that prevents the blood from clotting properly. Hemophilia A is inherited as a chromosome X-linked recessive disorder (Mannucci and Tuddenham, 2001) (Fig. 1.1).

There is currently no curative treatment for hemophilia A patients. The main treatment for hemophilia is called replacement therapy. Concentrates of plasma-derived or recombinant human FVIII is slowly injected into a vein. These infusions help replace the deficient or not enough FVIII. Plasma-derived FVIII is made from human plasma. Plasma donations are pooled together. Then, the plasma is fractionated into different products. One of the main products is the plasma-derived FVIII. Although the safety of plasma-derived products from the blood-borne transmission of viruses such as hepatitis virus or human immunodeficiency virus is greatly improved, there are still the uncertainty of the potential risk of viral transmission. Also, there are some limitations to produce the plasma-derived FVIII due to the limited supply of the human plasma. For this reason, the used amount of recombinant FVIII tends to increase recently.

The full length FVIII is an extremely large heterodimeric glycoprotein with a molecular weight of 330kDa (Fig. 1.2). The unprocessed FVIII contains 2332 amino acids, which are arranged in 6 domains (heavy chain, A1-A2-B and light chain, A3-C1-C2) (Bihoreau *et al.*, 1991).

FVIII undergoes processing prior to secretion into blood. It is released into the circulation as a set of heterodimeric proteins (Lenting *et al.*, 1998). The plasma purified FVIII is a metal ion complex of a light chain and a heavy chain. The light chain is

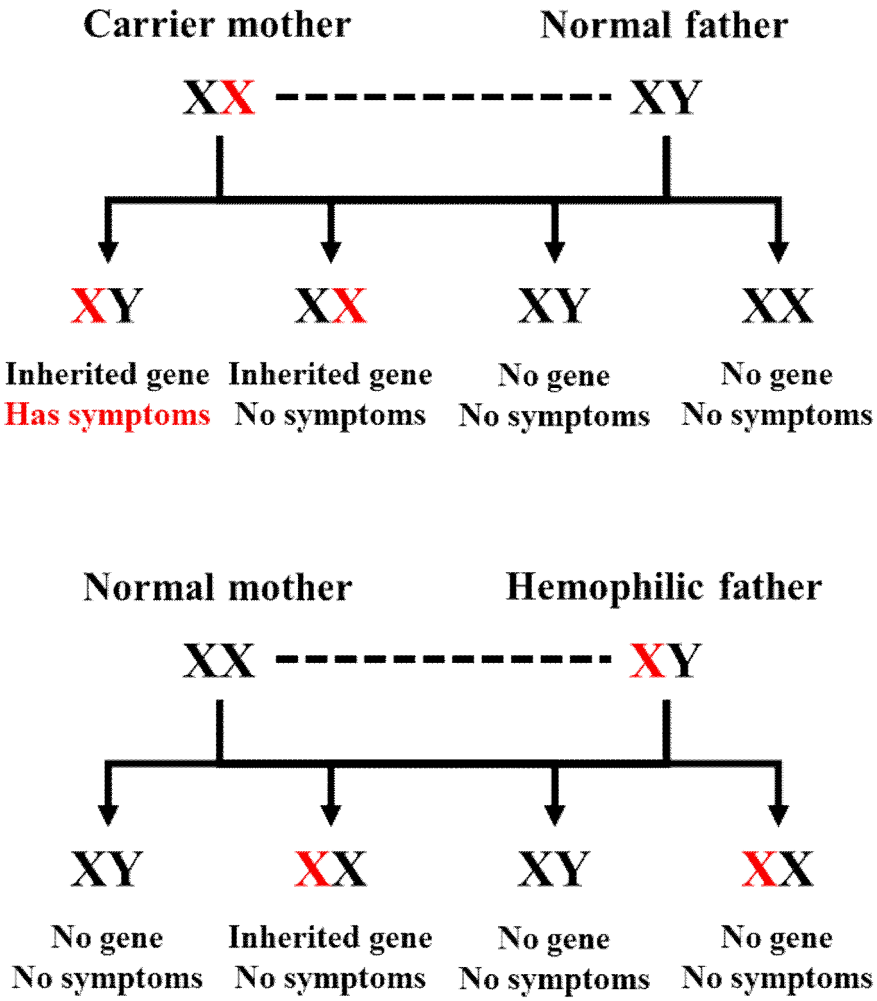


Fig. 1.1 Hemophilia transmission by a carrier mother and hemophilic father There are two ways the hemophilia gene can be transmitted. If the mother is a carrier, then 50% of her sons will have hemophilia and 50% of her daughters will also be carriers. If the father has hemophilia, his sons will be unaffected, as it is an X-linked disorder. All of his daughters will be carriers for the disorder. X: X chromosome, Y: Y chromosome, Red X: hemophilic X chromosome

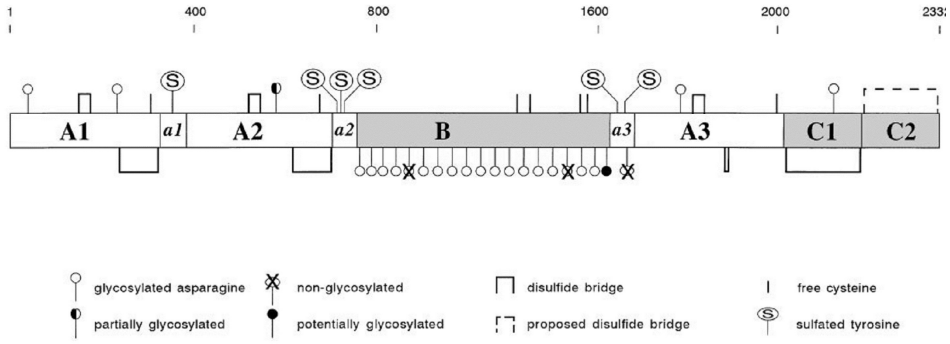


Fig. 1.2 Schematic model showing the domain structure of Factor VIII (Lenting *et al.*, 1998) Mature factor VIII consists of 2332 amino acids, which are arranged in a discrete domain structure: A1 (residues 1-336), A2 (373-710), B (741-1648), A3 (1690-2019), C1 (2020-2172), and C2 (2173-2332). The A domains are bordered by acidic regions a1 (337-372), a2 (711-740), and a3 (1649-1689).

composed of domains A3-C1-C2 with a molecular weight of 80kDa. The heavy chain has a molecular weight of 90-200kDa and is made up of domains A1-A2-B (Kaufman *et al.*, 1988).

FVIII exists FVIII/von Willebrand factor (vWF) complex in plasma before thrombin activation (Lenting *et al.*, 1998). The whole life span of FVIII is shown in Fig. 1.3. Upon activation by thrombin, FVIII is released from vWF and a series of specific proteolytic degradations occurs (Fig. 1.4). The active FVIII can participate in the intrinsic pathway for blood clotting (Fig. 1.5).

1.2 The instability of Factor VIII

Free FVIII is well known as a unstable protein in plasma as well as in a CHO cell culture supernatant (Bangham *et al.*, 1971; Kaufman *et al.*, 1988). In plasma, FVIII is usually complexed with vWF which is present in plasma in a large molar excess to FVIII. vWF binds to FVIII noncovalently at a constant ratio from 1:1 to 1:70 (Kaufman and Pipe, 1999; Vlot *et al.*, 1995). the vWF binding protects FVIII from inactivation by activated protein C and other proteases (Koppelman *et al.*, 1996).

Another plasma circulating protein, albumin, may also play a role in stabilizing FVIII in vivo. Currently marketed plasma-derived FVIII products and the first generation recombinant FVIII (rFVIII) therefore were stabilized by the addition of human albumin as a stabilizer during the manufacturing process and during storage (Boedeker, 2001; Gomperts *et al.*, 1992; Wang *et al.*, 2003).

However, because of the potential risk of pathogen contamination of plasma products such as human serum albumin, other excipients tend to be used as stabilizers such as sucrose or trehalose (Grillberger *et al.*, 2009). The second and third generation rFVIII products have albumin-free formulation for safety issues (Boedeker, 2001)(Table 1.1).

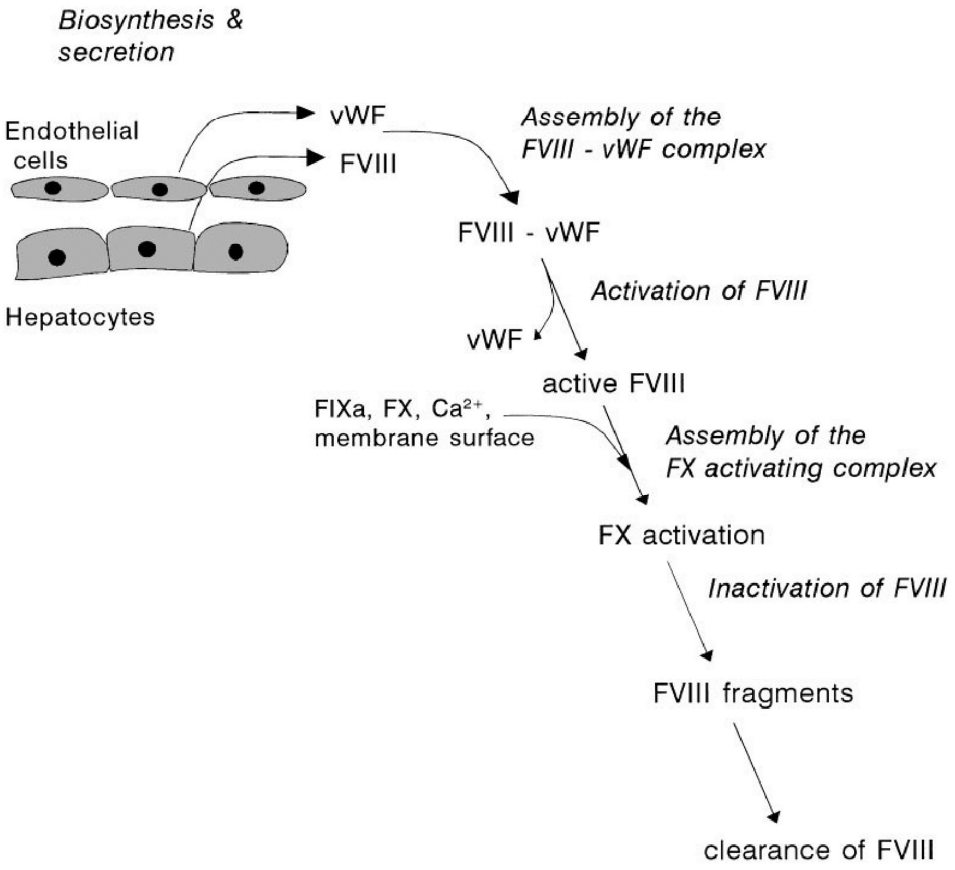


Fig. 1.3 The life span of Factor VIII (Lenting *et al.*, 1998) Factor VIII is synthesized by various tissues, including liver, kidney, and spleen, as an inactive single-chain protein. After extensive posttranslational processing, factor VIII is released into the circulation as a set of heterodimeric proteins. This heterogeneous population of factor VIII molecules readily interacts with vWF, which is produced and secreted by vascular endothelial cells. Upon triggering of the coagulation cascade and subsequent generation of serine proteases, factor VIII is subject to multiple proteolytic cleavages. These cleavages are associated with dramatic changes of the molecular properties of factor VIII, including dissociation of vWF and development of biological activity. After conversion into its active conformation, and participation in the factor X activating complex, activated factor VIII rapidly loses its activity. This process is governed by both enzymatic degradation and subunit dissociation.

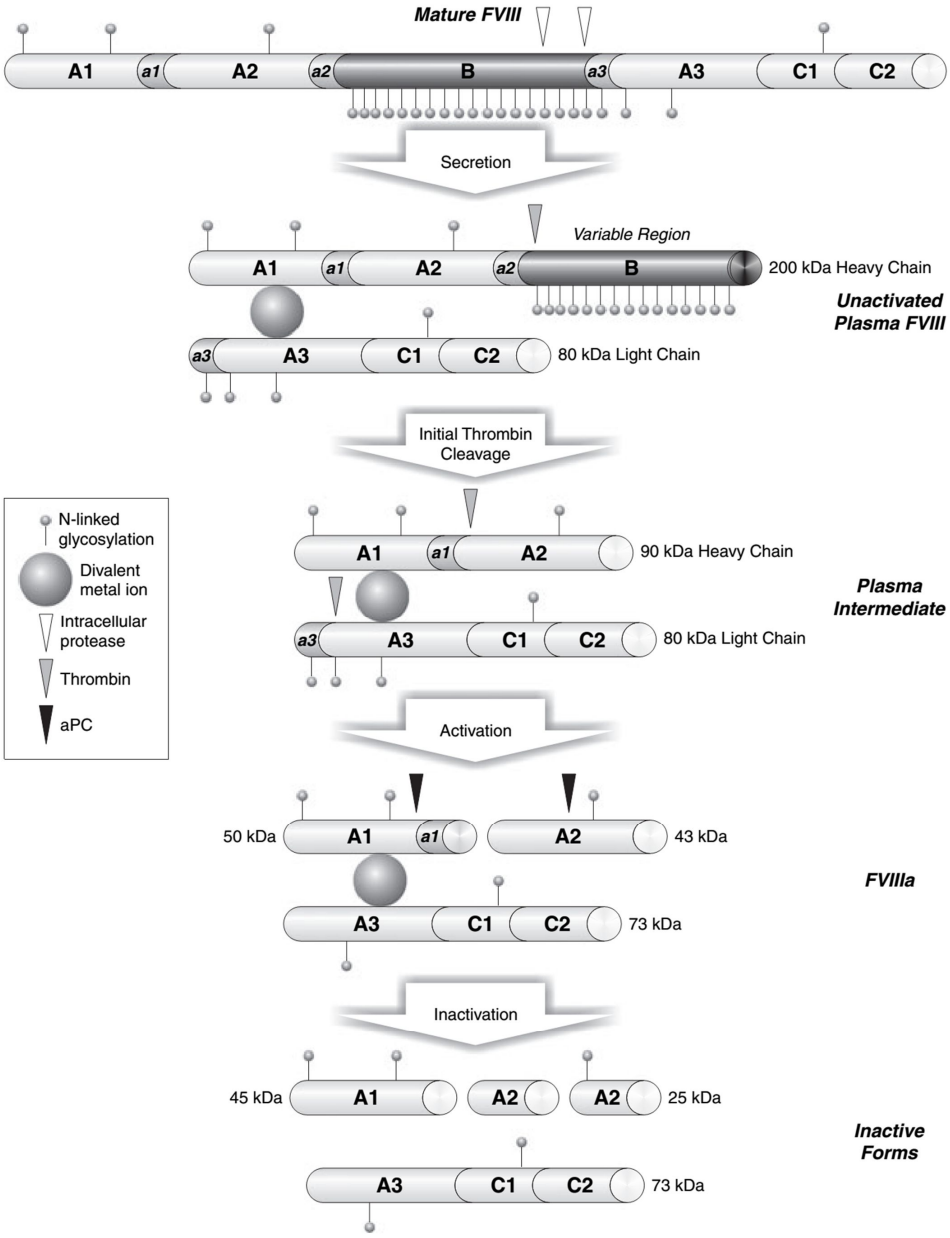


Fig. 1.4 Domain structure and processing of FVIII (Pipe, 2009)

aPC: activated protein C, FVIII: factor VIII, FVIIIa: activated factor VIII

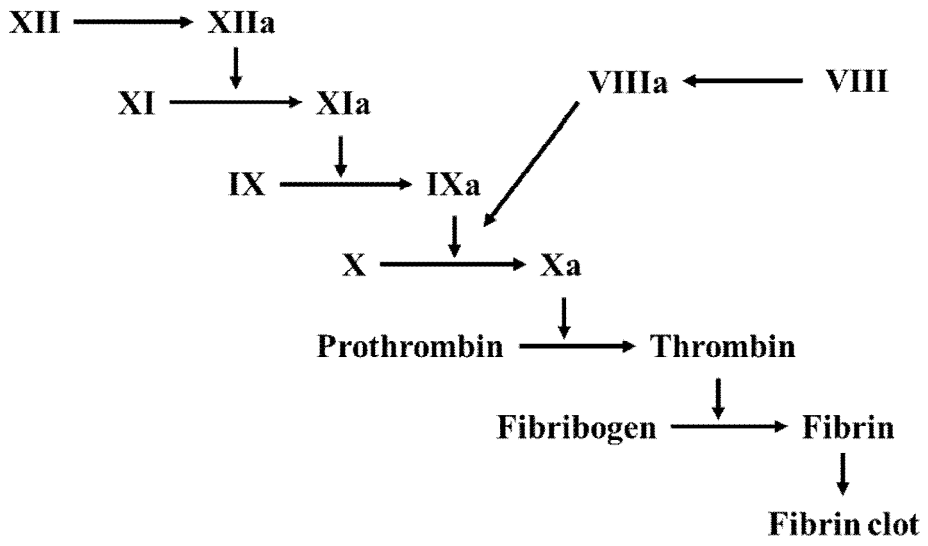


Fig. 1.5 Schematic representation of the blood clotting cascade XII: factor XII (Hageman factor), XIIa: activated XII, FXI: factor XI (deficient in hemophilia C), Xia: activated FXI, IX: factor IX (Christmas factor; deficient in hemophilia B), IXa: activated FIX, VIII: factor VIII (Brinkhous factor, deficient in hemophilia A), VIIIa: activated FVIII, X: factor X (Stuart-Prower factor), Xa: activated FX

Table. 1.1 Characteristics of commercially available FVIII products

	Brand name	Manufacturer	Stabilizer
Plasma-derived FVIII	Hemofil M	Baxter	Albumin
	Koate-DVI	Kedrion Biopharma	Albumin
	Monoclate-P	Baxter	Albumin
Recombinant FVIII (full length)	First generation		
	Recombinate	Baxter	Albumin (BSA used in culture medium)
	Second generation		
	Helixate FS	CSL Behring	Sucrose (human plasma protein solution used in culture medium)
	Kogenate FS	Bayer	
	Thrid generation		
	Advate	Baxter	Trehalose
Recombinant FVIII (B-domain deleted)	Xyntha	Wyeth	Sucrose

1.3 The B-domain of Factor VIII

The B-domain of FVIII is normally processed intracellularly at various positions to generate a heavy chain (A1–A2–B) varying from 90 to 200 kDa in size. Two of the domains, A and C, have been shown to share amino acid sequences similar to another factor protein (Factor V) and are thought to be essential to the coagulant activity of FVIII (Pipe, 2009).

However, the amino acid sequence of domain B is dissimilar to other factor protein. Although the role of domain B is not fully understood, it is not thought to be directly involved with coagulation and likely associated with intracellular processing of the protein (Pipe, 2009; Toole *et al.*, 1986).

Table 1.2 summarizes the reported function of the B domain. It was reported that deletion of this domain results in a smaller protein with no loss of coagulation activity and may be efficacious in treatment of hemophilia A in humans (Lind *et al.*, 1995; Pittman *et al.*, 1993; Sandberg *et al.*, 2001).

The smallest FVIII found in plasma is devoid of the heavily glycosylated B-domain. It is composed of a 90kDa heavy chain and an 80kDa light chain (Fig. 1.4). Recently developed recombinant FVIII, Refacto, is a FVIII preparations similar to the smallest FVIII protein found in plasma-derived FVIII concentrates. In case of Refacto, the B-domain is deleted and replaced with the SQ link of 14 amino acids (SFSQNPPVLKRHQR) between the A2 and A3 domains (Sandberg *et al.*, 2001) (Fig. 1.6). One of the benefits of the deletion of the B-domain is the higher expression level of recombinant FVIII in comparison to full-length FVIII (Toole *et al.*, 1986).

In this thesis, the B-domain deleted FVIII for the production of recombinant FVIII was used in order to increase the expression level of FVIII. The B-domain deleted FVIII of this thesis contains the SQ link of Refacto between the A2 and A3 domains.

Table 1.2 Functions of the B domain in the life cycle of FVIII (Pipe, 2009)

Process	Function	References
Intracellular		
Synthesis quality control	Enables interaction of factor VIII with chaperone proteins that distinguish properly folded tertiary structure of proteins; stabilizes folded domains, prevents aggregation	Pipe *et al.* (1998)
Secretion	Interacts with cargo-specific sorting receptor complex that enables endoplasmic reticulum to Golgi transport; increases secretion efficiency	Cunningham *et al.* (2003) Moussalli *et al.* (1999) Miao *et al.* (2004) Pipe *et al.* (2005) Zhang *et al.* (2005)
Plasma		
Activation	Possibly shields thrombin activation site from premature proteolysis	Eaton *et al.* (1986) Meulien *et al.* (1988) Pittman *et al.* (1993) Pittman *et al.* (1994)
Platelet binding	Decreases the affinity of unactivated factor VIII for activated platelets, thus preserving circulating factor VIII	Li and Gabriel (1997)
Inactivation	Reduces proteolysis by activated protein C and factor Xa	Khrenov *et al.* (2006)
Clearance	May play a further role in factor VIII quality control through interaction with asialoglyprotein receptor	Bovenschen *et al.* (2005)

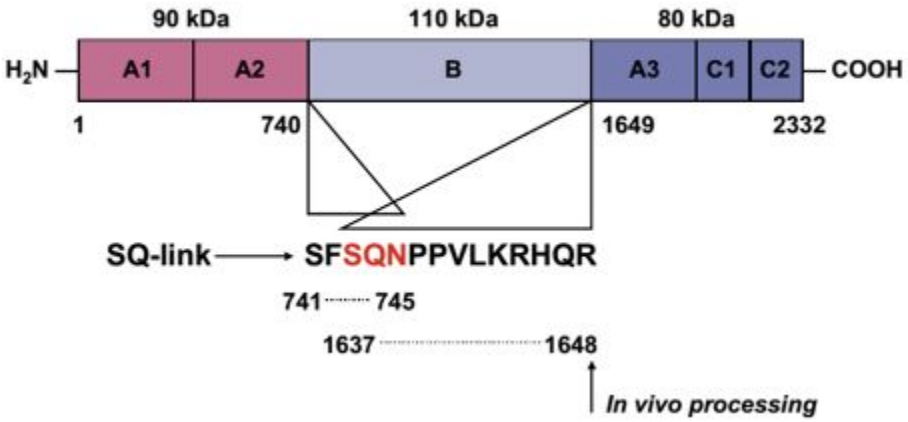


Fig. 1.6 The B-domain deleted FVIII molecule and the SQ link Full-length FVIII is a glycoprotein with the domain structure A1-A2-B-A3-C1-C2. (Pollmann *et al.*, 2002)

1.4 von Willebrand factor

von Willebrand Factor (vWF) is a blood glycoprotein with a molecular weight of 260 kDa as a monomer (Terraube *et al.*, 2010) (Fig. 1.7). vWF forms large multimers, sizing up to 20,000 kDa via *N*-terminal disulfide bridges (Choi *et al.*, 2007; Furlan, 1996) (Fig. 1.8).

vWF plays a central role in hemostasis. vWF performs its hemostatic function through binding to FVIII, to platelet surface glycoproteins, and to constituents of connective tissue. In primary hemostasis, vWF initiates platelet aggregation via binding to exposed structures of injured vessel walls at physiological high arterial shear rates (Sadler, 1998).

In secondary hemostasis, vWF supports platelet aggregation because of its binding to FVIII (Fang *et al.*, 2007), therefore protecting the coagulation factor from rapid proteolytic inactivation. Furthermore, vWF is thought to assist during platelet aggregation by bridging adjacent platelets at high shear rates.

The function of VWF is strongly shear rate dependent, whereas fluid dynamic conditions as well as mechanical forces are crucial for the conformational transition of vWF to develop its interaction with endothelial matrix proteins as well as platelets in case of vessel injury (Fig. 1.8).

A deficiency or abnormality of vWF results in the autosomally linked bleeding disorder known as von Willebrand disease (vWD) (Ruggeri and Zimmerman, 1987). vWF plays a crucial role in regulating FVIII concentration as well as FVIII:C in plasma (Wise *et al.*, 1991). vWF also functions as a carrier protein and stabilizer for FVIII protecting it from rapid proteolytic inactivation (Vlot *et al.*, 1995).

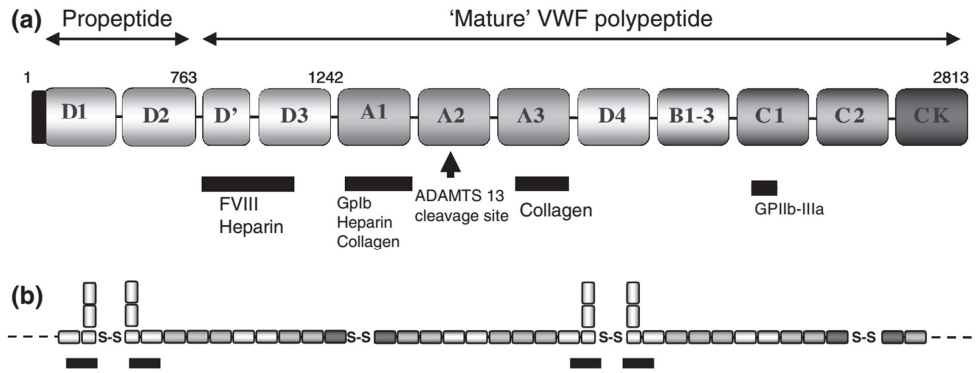


Fig. 1.7 Schematic representation of the pro-polypeptide and multimeric vWF. (a) the vWF molecule consists of 4 repeated domains A, B, C and D, the D1-D2 domains encompassing the propeptide. Ligand interaction sites are labelled. (b) vWF multimers are formed by C-terminal dimerization, followed by N-terminal multimerization, resulting in a head-to-head and tail-to-tail arrangement of monomers and juxtaposition of the FVIII binding region. (Terraube *et al.*, 2010)

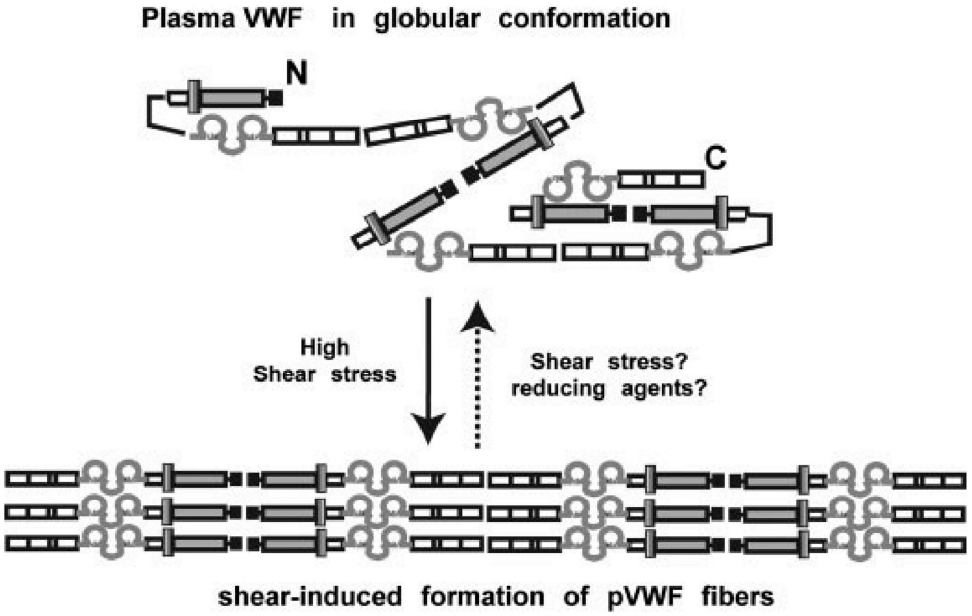


Fig. 1.8 Schematic illustration of vWF multimers. Upon exposure to high shear stress, globular vWF multimers are stretched to covalently associate with each other by forming interchain disulfide bonds. (Choi *et al.*, 2007)

1.5 Production of Factor VIII

Commercially available rhFVIII products have been produced including full-length rhFVIII (FL-rhFVIII) and B-domain deleted rhFVIII (BDD-rhFVIII) in either Chinese hamster ovary (CHO) cells or baby hamster kidney (BHK) cells (Boedeker, 2001). Recently Octapharma AG announced the availability of rhFVIII produced in human embryonic kidney cell line, HEK293 (Casademunt *et al.*, 2012).

The major drawback of rhFVIII production techniques is the low expression level due to the large molecular size and structural complexity of FVIII. There are several methods to overcome a low expression level, such as deletion of B-domain of FVIII, co-expression of the von Willebrand factor (vWF)(Berntorp, 1997; Boedeker, 2001; Jiang *et al.*, 2002; Kaufman *et al.*, 1988; Kaufman and Pipe, 1999; Weiss *et al.*, 1977), use of HKB11 cell as a host cell (Mei *et al.*, 2006), codon optimization of human FVIII cDNAs (Ward *et al.*, 2011), bioengineering of FVIII through elimination of a dispensable disulfide loop (Selvaraj *et al.*, 2012), development of lentiviral vectors (Mufarrege *et al.*, 2014) and over-expression of HSP70 (Ishaque *et al.*, 2007).

In this thesis, CHO cells co-expressing BDD-rhFVIII and vWF as a stabilizer to overcome the low expression level was used. In terms of culture mode, perfusion culture is more preferable over batch or fed-batch culture because of a higher instability of FVIII in the cell culture supernatant (Boedeker, 2001; Kelley *et al.*, 2010; Weiss *et al.*, 1977). Among the various cell retention devices, perfusion cultures with an external cell retention device are mainly used in the industrial production of the licensed rhFVIII preparations (Boedeker, 2001).

1.6 Perfusion culture

Batch, fed-batch and perfusion culture processes are commonly used in the

industrial-scale production of recombinant proteins and antibodies (Castilho and Medronho, 2002). Perfusion culture is not a up-to-date technique, because it has been already used in 1912 (Burrows, 1912) to keep small pieces of tissue viable for extended microscopic observations. The perfusion mammalian cell culture process has been typically used as a manufacturing process of unstable recombinant proteins, such as recombinant clotting factors, enzymes used in enzyme replacement therapy and other proteins (Chu and Robinson, 2001; Pollock *et al.*, 2013). The purpose of perfusion cell culture is the enhancement of volumetric productivity by achieving high cell density as compared with batch and fed-batch culture methods (Ryll *et al.*, 2000).

The principle of perfusion culture is a cell culture technique characterized by continuous flow of culture medium through the bioreactor with the retention of cells in the bioreactor (Fig. 1.9). Fresh medium is continuously added to supply various nutrients for cell growth and target protein production. Same amount of cell-free supernatant is withdrawn to remove harmful byproducts such as lactate, ammonia and to harvest target proteins. The key to successful perfusion culture is the retention of cells in the bioreactor by various cell retention devices. A number of cell retention devices are used to separate cells from the culture supernatants in perfusion culture (Fig. 1.10). Most cell retention devices are based on filtration, gravitational sedimentation, centrifugation (centrifuges, hydrocyclones) and ultrasonic separation.

There are many cell retention devices based on filtration process, such as cross-flow filter, vortex-flow filter, spin-filter and hollow fiber filter (Voisard *et al.*, 2003). A spin-filter and a Centritech Lab III system are traditional perfusion devices that have been widely used for several decades. ATF system is newly developed device for cell retention by Refine technology. In this thesis, these three devices were evaluated for the production of FVIII.

Table 1.3 Commercially available recombinant FVIII products (Boedeker, 2001)

Trade name	License holder	Manufacturing company	Cell line	method
Kogenate®	Bayer	Bayer	BHK	Perfusion
Helixate®	Aventis Behring	Bayer	BHK	Perfusion
Recombinate®	Baxter	Baster, Genetic Institute	CHO	Batch refeed
ReFacto®	Wyeth-Ayest	Genetic Institute, Pharmacia	CHO	Perfusion
KOGENATE® Bayer/Kogenate FS®	Bayer	Bayer	BHK	Perfusion

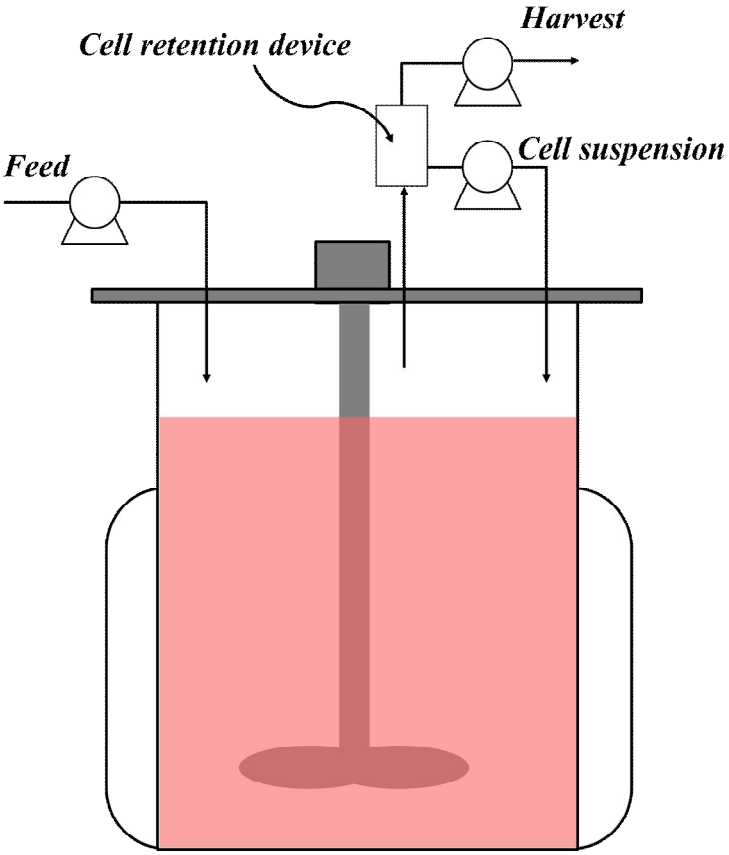


Fig. 1.9 Schematic representation of perfusion culture process

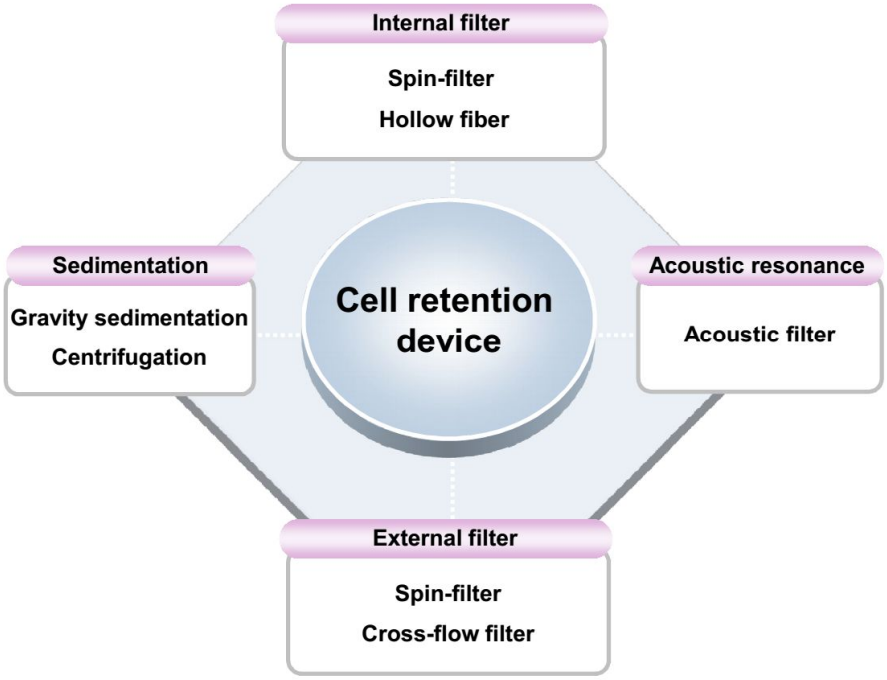


Fig. 1.10 Schematic diagram of the commonly used cell retention devices

1.6.1 Spin-filter

The most common perfusion systems used in commercial processes are spin-filters (Yabannavar *et al.*, 1992). Spin-filters are commercially available either as internal or external devices. Spin-filters was introduced as a cell retention devices for high density cell culture of murine leukemia cells in perfusion culture mode in the late 1970s by Himmelfarb *et al.* (1969). Spin-filter is a cylindrical membrane or stainless steel mesh of defined pore size. It is mounted on the impeller shaft and placed inside stirred tank bioreactor (Fig. 1.11). Fresh medium is pumped into the bioreactor, i.e. outside the spin-filter and cell-free supernatant is pumped out from inside the spin-filter at the same rate. In a spin-filter, cells are separated according to the mesh size of the spin-filter (Himmelfarb *et al.*, 1969; Yabannavar *et al.*, 1992).

1.6.2 Centritech Lab III centrifuge

Another commercially available and widely used cell retention device is a Centritech Lab III centrifuge manufactured by the PneumaticScaleAngelus (Fig. 1.12). The cell separation by a Centritech Lab III system is based on sedimentation caused by centrifugal force (Johnson *et al.*, 1996; Kim *et al.*, 2008; Tokashiki *et al.*, 1990). Centritech Lab III system consists of three parts, centrifuge unit, controller unit and pump unit. In Centritech Lab III system, cell-containing supernatant is fed into an inlet at the top of one end of the insert and the cell are centrifugally separated from the media. Clarified supernatant exists from an outlet at the top of the other end and cell concentrate is discharged from an outlet at the bottom of the insert (Fig. 1.13). Centritech Lab III centrifuge provides a relatively low-shear condition for cell separation compared with other cell retention devices.

Fig. 1.11 Spin-filter perfusion system

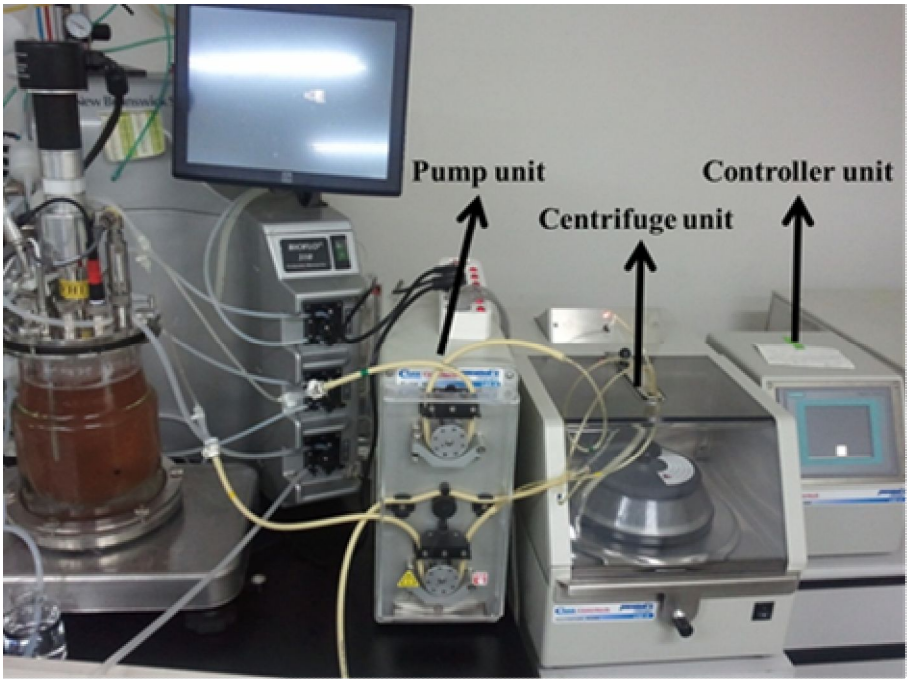


Fig. 1.12 Centritech Lab III centrifuge system

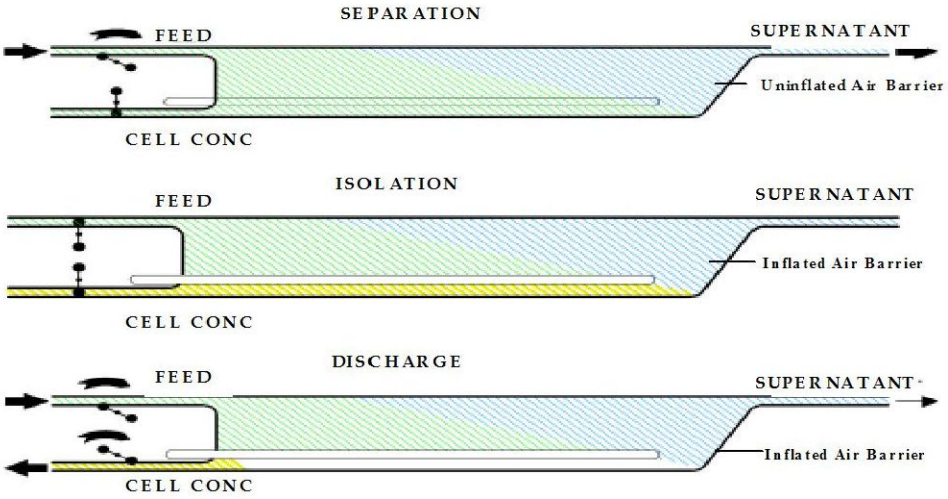


Fig. 1.13 The principle of cell separation in Centritech Lab III centrifuge Separation takes place in a pre-sterilized insert. Separation cycle: cells are separated under low g-forces while supernatant is continuously collected. Isolation cycle: concentrated cells are isolated by inflating a sealed air barrier which lays behind the separation insert cutting off the feed from the concentrated cells. Discharge cycle: concentrated cells (yellow) are discharged via the bottom pump

1.6.3 ATF system

Recently, an alternating tangential flow (ATF) system was utilized as a cell retention device. The ATF system from Refine Technology is the newest perfusion device using hollow fiber filter for cell separation from the spent medium (Crowley *et al.*, 2012; Shevitz, 2003). The ATF System provides an efficient means for fractionating a mixture or suspension of cells, molecules or other particulates. ATF is a patented system with flow pattern of changing tangential flow through a filter that results in a more efficient filtration process.

The ATF system consists of the controller, pump, housing, filtration device, and joint assembly (connects the housing to the vessel) (Fig. 1.14). The pump is partitioned by a medical grade flexible diaphragm into two compartments, an Air (**A**) side and Liquid (**L**) side. Any change in volume in compartment **A** will cause an equivalent but opposite change of volume in the **L** compartment. Controlled flow of filtered air to or from compartment **A** provides the driving energy for the corresponding, but inverse, liquid flow in other compartment B, or between a vessel and ATF Pump through a filter module. In the ATF system, cells are separated by repeated and rapid flow between the bioreactor and the hollow fiber filter due to the action of a diaphragm pump (Fig. 1.15).

The main features of the ATF system are self-cleaning and back-flushing action by a diaphragm pump (Fig. 1.16). The repeated and rapid flow between hollow fiber inlets and bioreactor by a diaphragm pump inhibits the blockage of hollow fiber inlets. In addition, back-flushing flow created by changing transmembrane pressure protects the hollow fiber filter from biofilm formation on its surface. These two main actions allow high cell density culture and extended perfusion time in ATF perfusion culture (Bonham-Carter and Shevitz, 2011; Furey, 2000; Furey, 2002).

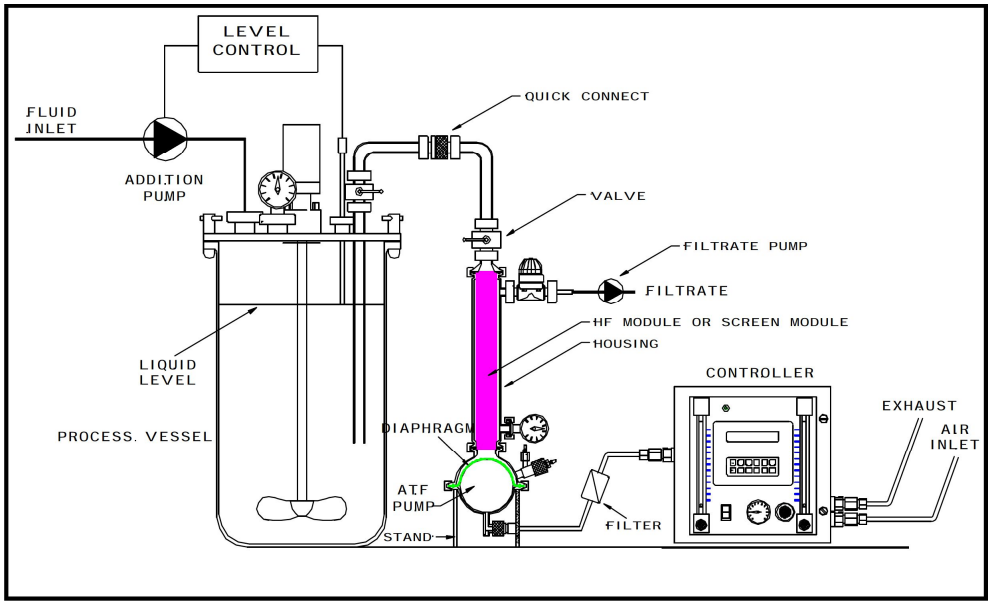


Fig. 1.14 Schematic view of ATF system (Furey, 2000)

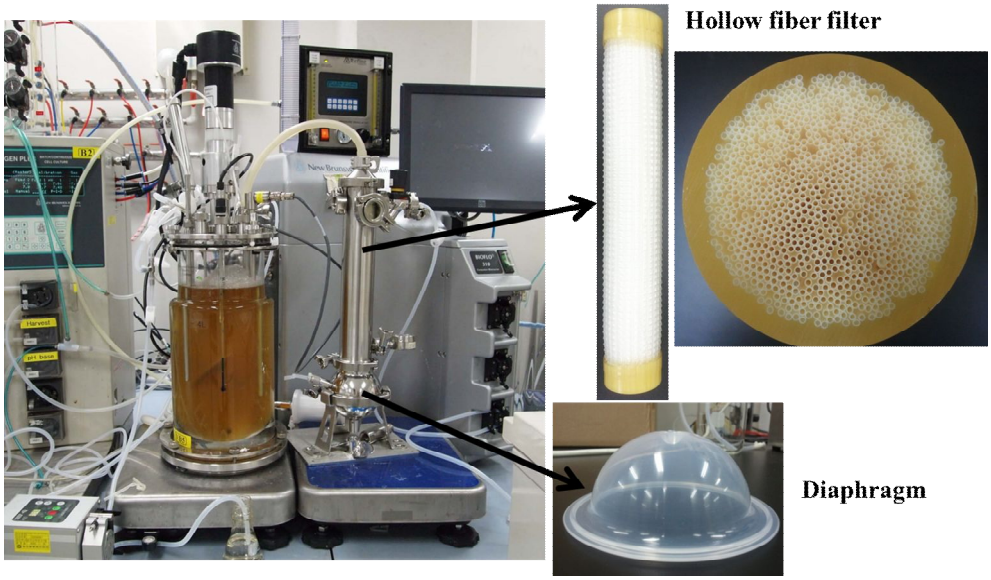


Fig. 1.15 ATF-4 system, hollow fiber filter and diaphragm

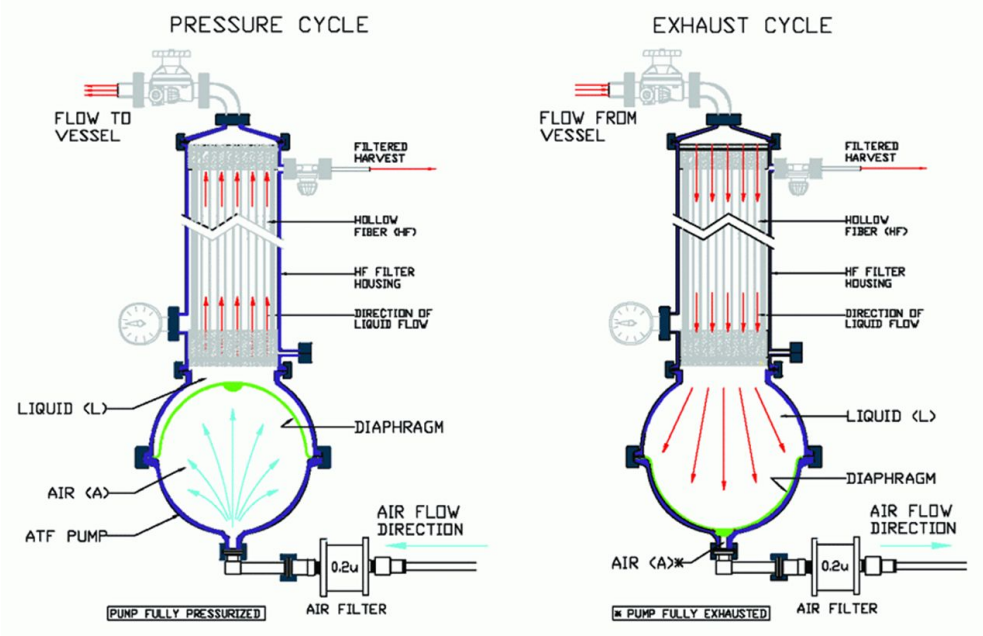


Fig. 1.16 The principle of diaphragm pump Pressure cycle - **A** Pressurizes by inward flow of air = Medium is expelled from **L**, through the HF Filter, to Vessel. 2. Exhaust cycle - **A** Exhausts by expulsion of air = **L** Expands by inflow of medium, through the Filter, form Vessel. (Sourced from www.knbs.co.kr)

1.7 The scope of thesis

The first purpose of this thesis is to investigate the reasons for the low FVIII:C recovery yield in the ATF perfusion culture. Three different perfusion cultures were performed to determine which of the three cell retention systems is the most suitable for producing BDD-rhFVIII co-expressed with vWF. Spin-filter, Centritech Lab III system, and ATF system were evaluated for their use as a perfusion device. Unexpectedly, it was found that the FVIII:C recovery yield is significantly low in only the ATF perfusion culture in the first study.

In filtration-based perfusion, such as ATF perfusion, the retention of the target protein inside the bioreactor due to a filter fouling is an inherent problem. Filter fouling occurs mainly because of the deposition of cell debris and nucleic acids in the perfusion culture of mammalian cells (Mercille *et al.*, 1994).

Although the ATF system is designed to minimize filter fouling by diaphragm pump action (Fig. 1.16), fouling problems were also observed in ATF perfusion culture. The concentration difference of high molecular weight antibody across the membrane surface was observed in a hollow fiber bioreactor, although a back-flush was applied, as in the ATF system. The retention rate was up to 20% (Hiller *et al.*, 1993). Others reported that up to approximately 60% of the produced antibody was retained by hollow fiber filter in ATF perfusion culture (Clincke *et al.*, 2013).

However, such a low yield of the target protein observed in the first study has not been reported during the production of other therapeutic proteins and antibodies in ATF perfusion cultures. Therefore, the possible factors that may be responsible for the low FVIII:C recovery yield was studied. Especially, the relationship between TMP across the hollow fiber filter membrane and FVIII:C recovery yield was examined. It was found out that transmembrane pressure has a great effect on the FVIII yield in ATF perfusion culture.

It was described in Chapter III.

The second purpose of this thesis is to develop a modified harvest system in order to increase the FVIII yield in ATF perfusion culture, which was described in Chapter IV. The system developed in this thesis consists of a check valve, pinch valve and timer (Fig. 4.1). The main idea is to increase the TMP across hollow fiber filter membrane, which was achieved by the use of the check valve. In Chapter V, the performance of the modified harvest system was compared with the peristaltic pump harvest system and examined the relationship between the enhanced FVIII:C recovery yield and the TMP across the hollow fiber membrane. It was reported that the modified harvest system achieved the higher TMP compared with a peristaltic pump harvest system. It was also demonstrated that the modified harvest system could be successfully applied to ATF perfusion culture instead of a peristaltic pump. In conclusion, the modified harvest system can achieve the enhancement of the concentration and product yield of FVIII:C.

The modified harvest system developed in this thesis can be applied to ATF perfusion culture for the production of other therapeutic proteins. The results of this thesis can give clues to solve a fouling problem in the filtration-based perfusion device.

Chapter II

Materials and Methods

2.1 Cell line and inoculum preparation

The cell line used was generated from dihydrofolate reductase (DHFR)-deficient Chinese hamster ovary (CHO) cells (DG44; obtained from Dr. L. Chasin). CHO cells were transfected with an expression vector encoding B-domain deleted rhFVIII (BDD-rhFVIII). Then a vWF expression vector was introduced into the BDD-rhFVIII producing CHO cells (Fig. 2.1). The inoculum for the bioreactor culture were produced using Erlenmeyer flasks (Corning, USA). Erlenmeyer flasks were maintained at 100 rpm on an orbital shaker in a humidified 5% CO_2 incubator (Sanyo, Japan) at 34°C. Both in an Erlenmeyer flask and bioreactor, cells were cultured in a CHO DHFR- medium (SAFC biosciences, USA) supplemented with glutamine. Cells were passaged twice a week by dilution with fresh medium.

2.2 Perfusion culture

Three different cell retention devices, a Centritech Lab III centrifuge (Carr Centritech, USA), a spin-filter (New Brunswick Scientific, NJ), and an ATF-4 system (Refine Technology, NJ) were used for perfusion culture. Perfusion cultures were performed in a 5 L BioFlo 310 bioreactor (New Brunswick Scientific, NJ) with a working volume of 3L.

The ATF-4 system used was controlled by the C24-4 version 1.01 controller from Refine Technology. A polyether sulfone (PES) hollow fiber filter cartridge (Refine Technology, NJ) for ATF-4 system with a 0.2 μm (F4:RF-02PES, Refine Technology) or 0.5 μm (F4:RF-05PES, Refine Technology) pore size was used for ATF perfusion.

A Centritech Lab III centrifuge was operated in the intermittent pump mode for perfusion. Operating parameters such as separation time, discharge time, separation speed, discharge speed, rotor speed, feed flush time, concentrate flush time, final flush time,

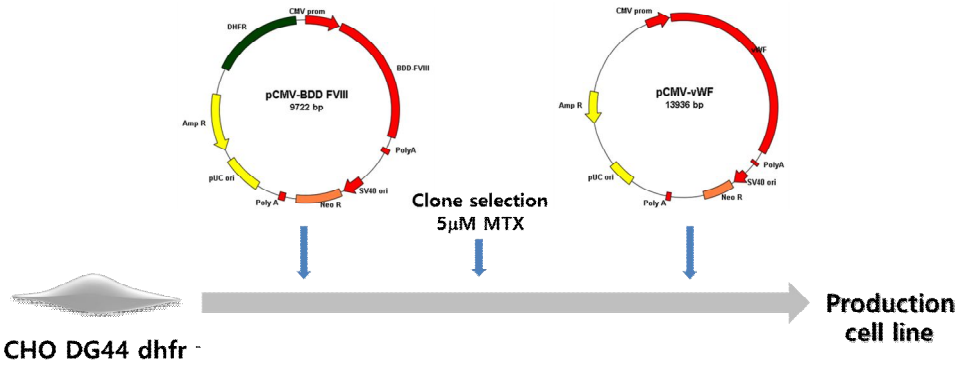


Fig. 2.1 Establishment of the production cell line co-expressing FVIII and vWF the BDD-FVIII expression vector (pCMV-BDD FVIII) was introduced into DG44 CHO cell line with a dihydrofolate reductase (DHFR) gene. After methotrexate (MTX) amplification, the FVIII producing CHO cell line resistant to 5μM MTX was selected. Then, the vWF expression vector (pCMV-vWF) was introduced into the FVIII producing CHO cell.

waiting time, and number of cycles were set at 6 sec, 5 sec, 38 rpm, 30 rpm, 800 rpm, 46 sec, 37 sec, 60 sec, 40 min, and 39 cycles, respectively, at a perfusion rate of 2 vvd (volume of medium perfused per bioreactor volume per day).

An internal spin-filter (#M1273-3202, New Brunswick Scientific, NJ) was used with a BioFlo 310 bioreactor for perfusion culture. The spin-filter module had a filter screen with 12-14 μm mesh size and a low shear marine blade impeller.

The same culture parameters, as described below, were used for the three perfusion cultures. The seed cells grown in Erlenmeyer flasks were inoculated in the BioFlo 310 bioreactor. The bioreactor was equipped with a dissolved oxygen (DO) probe, pH probe, and temperature probe. Culture temperature, DO, pH, and agitation speed were controlled automatically and monitored using BioCommand software (New Brunswick Scientific, JN) and a personal computer.

The DO concentration was kept at approximately 50% of air saturation by adjusting the air/O2 ratio of the inlet gas. The agitation speed was also varied between 100 to 150 rpm to maintain the DO level. The pH was kept at a constant value of 7.0 by adding a 7.5% (w/v) $NaHCO_3$ solution or adjusting the CO_2 ratio of the inlet gas. The culture temperature was maintained at 34°C.

Approximately 30 mL of the samples were taken once every day after the perfusion was started. After centrifugation for cell separation, an aliquot of the sample was immediately frozen at -80°C for further analysis.

Glucose, lactate, glutamine, and ammonia concentrations were analyzed off-line by a YSI 7100 multiparameter bioanalytical system (YSI Inc., Yellow Springs, OH).

Cell density and viability were measured by an automated trypan blue cell density examination system (Cedex, Innovatis GmbH, Germany). Viable cell density was stably maintained by cell bleeds. The perfusion rate was controlled by adding fresh

medium and withdrawing an equal volume of clarified supernatant with a peristaltic pump.

Perfusion was started when the residual glucose level was below 1 g/L. The perfusion rate was adjusted in order to maintain glucose level above 1 g/L. A 20 L BioEaze bag (SAFC biosciences, USA) was used as a harvest bag. The harvest bag was placed in a 4°C refrigerator until replacement with a new harvest bag.

2.3 Dead-end filtration

The harvested supernatant from both Centritech Lab III and spin-filter perfusion was filtered by a Millipak 200 filter (Merck Millipore, USA) with 0.2 μm pores. Before first use, the filter was wetted out by flowing distilled deionized water with a peristaltic pump. After filtration of 5 L supernatant, the FVIII:C in the feed sample and filtrate was measured by a one-stage clotting assay.

2.4 Storage and surface adsorption experiments

The stability of FVIII was assessed at three different temperatures: 4°C, -20°C and -80°C. Centrifugation was performed for 4min at 3000rpm using Centrifuge 5810R (Eppendorf, Germany) to clarify the supernatant from the ATF perfusion culture. The clarified supernatant containing FVIII was stored in triplicate 1 mL aliquots in polypropylene tubes at each temperature. The FVIII:C was measured at 24h after storage.

The adsorption experiment was performed for two FVIII activities at 4°C. The FVIII activities of the high- and low-activity preparation were 2.1 AU and 0.115 AU, respectively. These preparations were obtained from the ATF perfusion culture. Each duplicate 12 L sample was stored at 4°C in the 20 L BioEaze bag (SAFC biosciences, USA), which was used as a harvest bag. The surface material of the BioEaze bag is a single layer of Dow ATTANE™, an ultra-low density polyethylene (ULDPE). The FVIII:C in the

supernatant was measured at times 0, 3, 6, 18, and 24h, respectively.

2.5 Controlled TMP experiment

A KrosFlo Research IIi tangential flow filtration system (Fig. 2.2) (Spectrum Laboratories, Inc., CA) was utilized to measure the transmembrane pressure across the MiniKros plus hollow fiber filter module (Spectrum Laboratories, Inc., CA) with a 0.2 μm pore size and 3100 cm^2 surface area. Polysulfone pressure transducers were connected to the inlet, the outlet, and the permeate port of the MiniKros plus hollow fiber filter module.

The culture supernatant from the Centritech Lab III perfusion was circulated through a MiniKros plus hollow fiber filter thru the built-in peristaltic pump of a KrosFlo Research IIi system. The TMP was controlled by the peristaltic pump speed and the KrosFlo automatic backpressure valve according to the manufacturer's instructions. Pressure changes at each point were monitored simultaneously using KF COMM software from Spectrum Laboratories. The TMP was calculated by using the following formula: TMP = (Inlet pressure + Outlet pressure)/2-Permeate pressure

2.6 Perfusion culture with a modified harvest system and a peristaltic pump system

The ATF-4 system and C-24 controller (both from Refine Technology) were used for perfusion culture. The seed cells were inoculated in a BioFlo 310 bioreactor (New Brunswick Scientific) at a starting concentration of approximately $5x10^5$ cells/mL. The working volume was 3 L. Hollow fiber modules (Refine Technology) with a 0.2 μm pore size was used. ATF flow rate was 3 L/min. One perfusion run (ATF no. 1) was data from our previous study (Kim *et al.*, manuscript submitted) using a peristaltic pump for harvest. Two perfusion runs were performed to evaluate the modified harvest system with the ATF

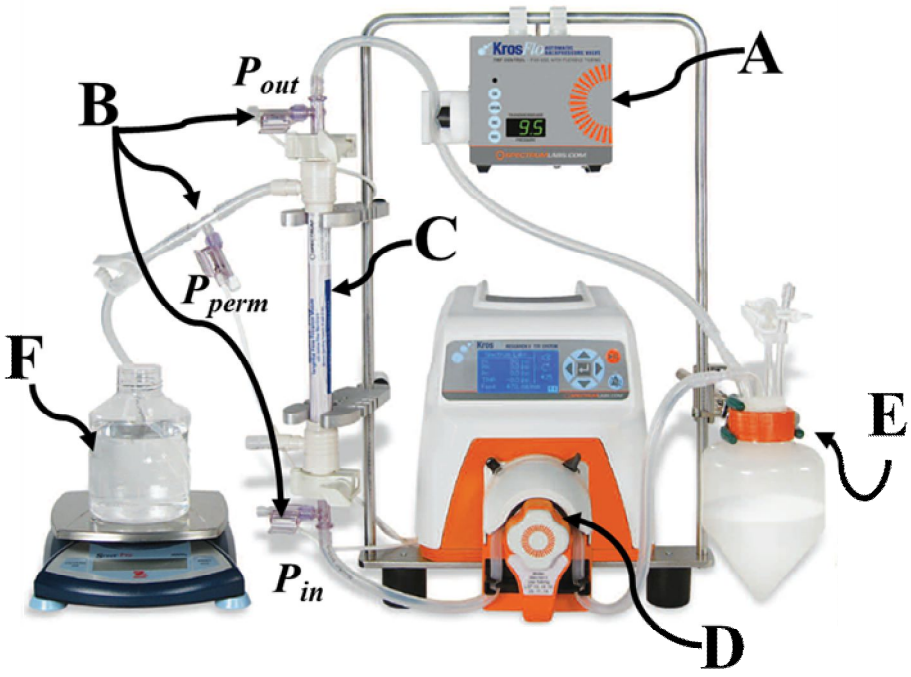


Fig. 2.2 KrosFlo Research IIi system (source: www.spectrumlabs.com) A: backpressure valve, B: pressure transducer, C: hollow fiber filter, D: peristaltic pump, E: retentate reservoir, F: permeate reservoir, P_{in}: inlet pressure, P_{out}: outlet pressure, P_{perm}: permeate pressure

system. Data from these two runs were averaged and are denoted ATF no. 2.

In a peristaltic pump harvest system, the perfusion rate was controlled as follows. The supernatant filtered through a hollow fiber filter was drawn into a harvest bag at a constant rate by the built-in peristaltic pump of the aforementioned bioreactor. Fresh medium was automatically added by the peristaltic pump, which was actuated by the weight change of the bioreactor vessel measured by a load cell until the vessel weight is increased to the set-point.

The system function was as follows. First, fresh medium was added to the bioreactor vessel at a constant rate by the built-in peristaltic pump. If the vessel weight increased to the set-point, the load cell provided an off signal to the timer connected to the pinch valve. The timer allowed the pinch valve to open for one minute, even though the vessel weight had decreased to below the set-point. The controller then provided an on signal to the pinch valve. The above cycle was repeated, making it possible to control the perfusion rate.

Daily samples were taken from the bioreactor for analysis. After centrifugation, the samples were immediately frozen at -80^{o}C for further analysis. The viable cell density and viability were measured by trypan blue staining using Cedex (Innovatis GmbH, Germany).

2.7 Measurement of TMP during the operation of ATF system

The bioreactor vessel was filled with deionized water instead of cells and medium for TMP measurement experiment under the cell-free condition. A KrosFlo Research IIi system (Spectrum Laboratories, CA) was used to measure TMP across the 0.2 μm pore size hollow fiber filter (Refine Technology).

As shown in Fig. 2.3, three pressure transducers were connected to the bioreactor

connection port, the second chamber of a diaphragm pump and the harvest port of the hollow fiber filter module. Pressure measurement at each point was performed using KF COMM software (Spectrum Laboratories).

TMP was automatically calculated as using the following formula: (Inlet pressure at the bioreactor connection port + Outlet pressure at the second chamber of a diaphragm pump) / 2 – Permeate pressure at the harvest port.

2.8 FVIII:C and antigen assay

FVIII:C was measured by a one-stage clotting assay with an ACL TOP 500 CTS (Instrumentation Laboratory, USA) according to the manufacturer's instructions and as per the Fourth International WHO standard. FVIII antigen content (FVIII:Ag, FVIII antigen) was quantified by an anti-FVIII light chain sandwich enzyme-linked immunosorbent assay (ELISA) using an in-house BDD-rhFVIII antigen as a standard and an in-house ELISA kit.

2.9 vWF antigen assay

vWF protein content (vWF:Ag, VWF antigen) was quantified by a sandwich ELISA, REAADS® von Willebrand factor antigen test kit (Corgenix Inc., Broomfield, CO, USA) according to the manufacturer's instructions.

2.10 Statistical analysis

Data were statistically analyzed by a two-tailed student’s t-test using Microsoft Excel. Statistical significance was defined as $P < 0.05$.

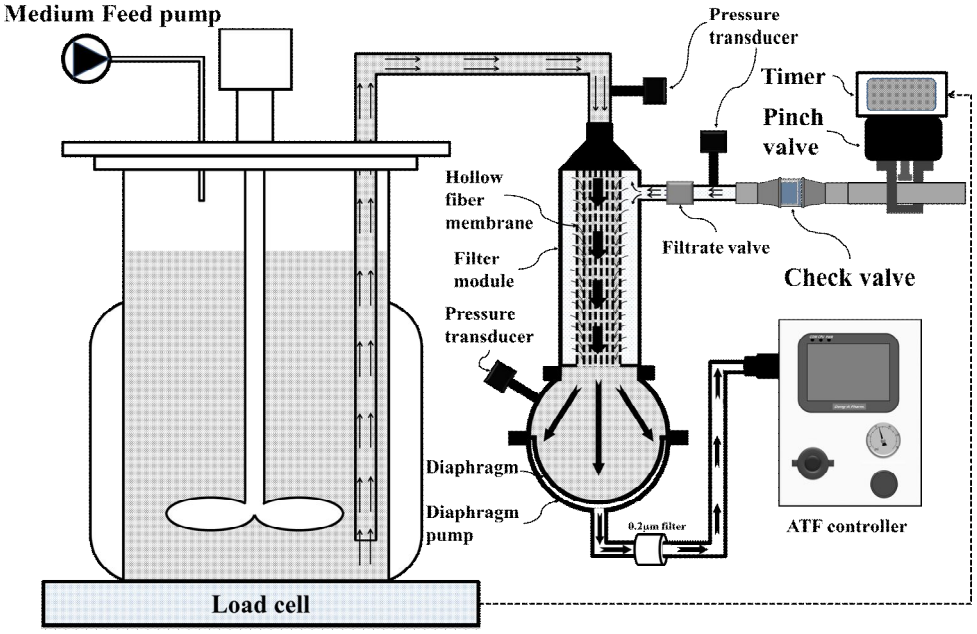


Fig. 2.3 TMP measurement during the operation of ATF system Pressure transducers are connected to KrosFlo Research IIi system for the real time measurement of transmembrane pressure

Chapter III

Study of the effect of transmembrane pressure on Factor VIII yield in ATF perfusion culture

3.1 Cell growth and FVIII production in three different perfusion cultures

Three different perfusion cultures were performed to compare performance in the production of BDD-rhFVIII co-expressed with vWF. Fig. 3.1A shows cell growth performance during perfusion culture using the three different devices.

In the Centritech Lab III perfusion culture, viable cell density was stabilized at an average of $3.5x10^7$ cells/mL at a perfusion rate of 2 vvd after day 10. Since the separation efficiency rapidly decreased above $4x10^7$ cells/mL, viable cell density was controlled below $4x10^7$ cells/mL.

Viable cell density in the spin-filter perfusion was maintained at an average of $1.76x10^7$ cells/mL for the culture duration after day 10 at a perfusion rate of 1.5 vvd in order to avoid filter clogging and extend culture duration.

In the ATF perfusion culture, viable cell density was stabilized at an average of $5.02x10^7$ cells/mL at a perfusion rate of 2 vvd. However, after day 27 viable cell density was increased to between 8 and $10x10^7$ cells/mL in order to evaluate the performance of the ATF system in a very high cell density.

The FVIII:C in the bioreactor varied between 0.1 to 0.2 AU in both the Centritech Lab III and spin-filter perfusion cultures (Fig. 3.1B). The average value of FVIII:C in the bioreactor was 0.138 AU and 0.131 AU in the Centritech Lab III and spin-filter perfusion cultures, respectively. The average FVIII:C in the harvest after cell density stabilization was 0.13 AU and 0.17 AU in the Centritech Lab III and spin-filter perfusion cultures, respectively (Fig. 3.1D). As shown in Fig. 3.1C, these two perfusion cultures showed more than a 70% FVIII:C recovery yield.

As shown in Fig. 3.1B-D, the FVIII production profile in the ATF perfusion culture was significantly different from the other two perfusion cultures. The FVIII:C in the bioreactor was rapidly increased up to 1.05 AU according to an increase in culture time

(Fig. 3.1B). In contrast, FVIII:C in the harvest was continuously decreased with an increase in the culture time, and FVIII:C in the final harvest bag was 0.024AU (Fig 3.1D). At the beginning of the ATF perfusion culture, the FVIII:C recovery yield was 71.2% (Fig 3.1C). This yield at the start of perfusion culture was almost equivalent to that in the Centritech Lab III or spin-filter perfusion cultures. However, FVIII:C recovery yield rapidly decreased over the culture time. The average FVIII:C recovery yield was only 8.2% in the ATF perfusion culture. This yield was 10-fold lower than that in the other two perfusion cultures. Such a low yield of the interest protein as well as FVIII in ATF perfusion culture, has not been previously reported.

3.2 Effect of storage temperature on FVIII:C recovery yield

Since the FVIII molecule is an unstable protein in a CHO cell culture supernatant as described previously (Kaufman *et al.*, 1988), there is a strong possibility that inactivation of FVIII may occur. Therefore, a FVIII storage stability test was performed to check whether FVIII inactivation occurs during storage in the harvest bag. A polypropylene tube was utilized as a storage container in order to exclude the problem of surface adsorption of FVIII molecules (McLeod *et al.*, 2000). When the perfusion rate was 4vvd, the harvest bag was replaced with a new harvest bag after every 24h. Therefore, if the FVIII:C had not decreased after 24h storage, it was obvious that the inactivation of FVIII was not a dominant factor in the reduction of the FVIII:C recovery yield.

As shown in Table 3.1, FVIII:C recovery after 24h storage at 4^{o}C was 92.8%. A slightly lower FVIII:C recovery was noted in the other two storage conditions, 86.9% for -20^{o}C (*$P < 0.05$) and 90.9% for -80^{o}C. Hence, this result implies that although inactivation of FVIII may occur during storage in the harvest bag, it is not a dominant factor that affects FVIII:C recovery yield.

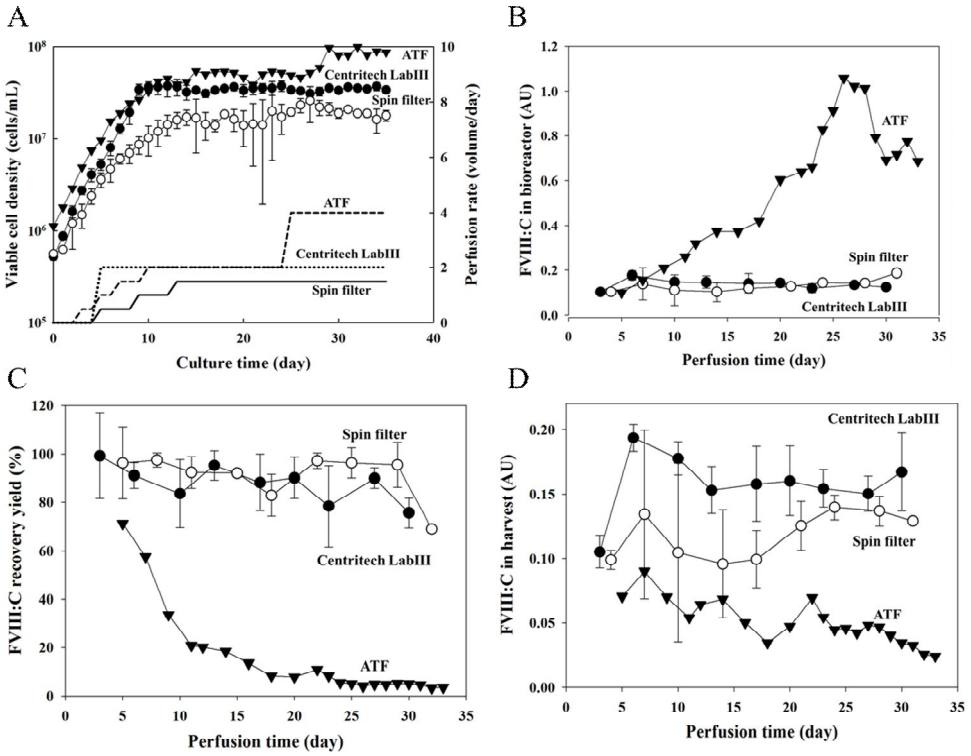


Fig. 3.1 Comparison of three cell retention devices. Cell growth performance (A), FVIII:C in the bioreactor (B), FVIII:C in harvest (C), and FVIII:C recovery yield (D). The FVIII:C in the bioreactor was calculated based on the average of FVIII:C measured in daily samples taken from the bioreactor until the next harvest. Error bars represent standard deviations. Number (n) of separate experiments: n=3 (Centritech Lab III), n=2 (spin-filter) and n=1 for ATF. AU indicates arbitrary unit.

Table 3.1 Comparison of FVIII:C and recovery after storage at various temperatures

Storage temperatures	Time = 0	4°C	-20°C	-80°C
FVIII:C (AU)[a]	2.03 ± 0.13	1.88 ± 0.09	1.76 ± 0.18	1.84 ± 0.11
Recovery (%)[b]	100	92.8	86.9*	90.9

[a] Mean ± SD (n=3), [b] Recovery (%) were calculated based on average FVIII:C. AU indicates arbitrary unit. * $P < 0.05$

3.3 Effect of the adsorption of FVIII molecule onto the container surface on FVIII:C recovery yield

McLeod *et al.* (2000) previously reported the loss of recombinant FVIII:C during storage in polyvinyl chloride (PVC) containers, but not in polypropylene syringes. They obtained a 41.9% and a 43.7% FVIII:C recovery in undiluted (146 IU/mL) and diluted (10 IU/mL) samples after 48h of storage in a PVC bag at room temperature. However, only 1.8% FVIII:C was obtained in FVIII diluted to 2IU/mL. Hence, in their study, they found that the loss of FVIII:C was due to the adsorption of FVIII:Ag onto the surface of PVC.

Their result suggests that the adsorption of FVIII:Ag can also be an important cause for low FVIII:C recovery yield in the ATF perfusion culture. Therefore, FVIII:C recovery yield after 24h storage at 4°C in the harvest bag was measured to check whether the loss of FVIII:C was due to FVIII:Ag adsorption onto the surface of the harvest bag.

When the perfusion rate was 4vvd, the harvest bag was replaced with a new harvest bag after every 24h. Therefore, if the FVIII:C had not decreased after 24h storage, it was obvious that the adsorption of FVIII was not a dominant factor in the reduction of the FVIII:C recovery yield.

Table 3.2 shows that the recovery of FVIII:C was 89% for 2.1 AU and 95.7% for 0.115 AU after 24h. This loss of activity could be due to the inactivation of FVIII during storage. Therefore, it was concluded that the loss of FVIII:C shown in Table 3.2 might be mostly due to the inactivation of FVIII during storage. It was also concluded that the adsorption of FVIII:Ag onto the surface of the harvest bag was not a very significant cause.

The results of Table 3.1 and Table 3.2 evidently showed that the adsorption of FVIII:Ag onto the surface of the harvest bag and inactivation of FVIII were not the main causes of a low FVIII:C recovery yield in the ATF perfusion culture.

Table 3.2 Variation in FVIII:C and recovery over time during storage in the harvest bag

Storage duration (h)	FVIII:C (AU)	
	High activity	Low activity
0	2.10 ± 0.04 (100)	0.115 ± 0.009 (100)
3	2.05 ± 0.05 (97.6)	0.117 ± 0.003 (102)
6	1.91 ± 0.06 (90.9)	0.109 ± 0.012 (94.8)
18	1.78 ± 0.12 (84.8)	0.106 ± 0.006 (92.2)
24	1.87 ± 0.08 (89.0)	0.110 ± 0.009 (95.7)

The values in parentheses indicate the FVIII:C recovery calculated based on average FVIII:C. AU indicates arbitrary unit. Mean± SD (n=2)

3.4 Effect of the retention of FVIII:Ag by the hollow fiber filter on FVIII:C recovery yield

In this study, it was assumed that three possible mechanisms could lead to a very low FVIII:C recovery yield in ATF perfusion culture. In the previous section, two possible mechanisms such as the inactivation and the adsorption of FVIII were investigated. It was also demonstrated that those factors were not a dominant factor of a low FVIII:C recovery yield in ATF perfusion culture.

The last possible cause of low FVIII:C recovery yield was the retention of FVIII:Ag by the hollow fiber filter membrane. Since fouling of the hollow fiber filter is an intrinsic problem of a membrane-based perfusion culture (Mercille *et al.*, 1994), the possibility of a reduction of yield due to fouling obviously exists even in an ATF perfusion culture.

Since most of the secreted FVIII exists as a FVIII/vWF complex in the supernatant of a CHO cell culture (Kaufman and Pipe, 1999; Wise *et al.*, 1991), a reduction in the amount of FVIII:Ag will be accompanied by a decrease in the amount of vWF:Ag. Moreover, the loss of co-secreted vWF:Ag will occur at the same rate, because vWF has the capacity to form a complex with FVIII at a constant ratio of 1:1 ~ 1:70 as described previously (Kaufman and Pipe, 1999; Vlot *et al.*, 1995). Therefore, FVIII:Ag and vWF:Ag assays was performed to assess the possibility of retention of FVIII:Ag due to the filtration action of ATF hollow fiber filter.

As shown in Table 3.3, the FVIII:C recovery yield at day 35 of ATF perfusion culture was 3.13%. The vWF yield by vWF:Ag ELISA and the FVIII:Ag yield by FVIII:Ag ELISA was 2.99% and 2.50%, respectively. The decreasing ratio of FVIII:Ag yield was quite similar to the ratio of the reduction of vWF:Ag yield. It was also very similar to the decreasing ratio of the FVIII:C recovery yield.

Table 3.3 Comparison of FVIII:C, FVIII:Ag, and VWF:Ag yield at day 35 in ATF perfusion culture

	Bioreactor (AU)	Harvest (AU)	Yield (%)
FVIII:C	0.775	0.025	3.13
FVIII:Ag	0.959	0.024	2.50
VWF:Ag	0.885	0.026	2.99

Yield (%) = Harvest (AU) / Bioreactor (AU) * 100. AU indicates arbitrary unit.

Since inactivation and protein loss by surface adsorption was shown to not be dominant factors in low FVIII:C recovery yield, these results clearly indicate that the retention of FVIII:Ag by the hollow fiber filter was the main cause of the reduction of FVIII:C recovery yield. Furthermore, these results also suggest that the increase in FVIII:C in the bioreactor during ATF perfusion culture was due to the accumulation of retained FVIII:Ag (Fig. 3.1B).

3.5 Retention of FVIII:Ag by dead-end filtration

The culture supernatant collected in the harvest bag in both the Centritech Lab III and spin-filter perfusion cultures must be clarified using a filter with a pore size smaller than 0.2 μm in order to proceed to the purification processes. Therefore, the actual FVIII:C recovery yield should be calculated after filtration of the harvest. Fig. 3.2 shows the FVIII:C recovery yield after filtration using a membrane with 0.2 μm pores for supernatants from both perfusion cultures. After membrane filtration, a decrease in FVIII:C recovery yield was observed. The FVIII:C recovery yield was 63.7% for the Centritech Lab III and 52.8% for the spin-filter. Although these yields were significantly higher than that in the ATF perfusion culture, this also provided clear evidence of the retention of FVIII:Ag by filtration action.

3.6 Effect of the pore size of hollow fiber filter on FVIII:C recovery yield

ATF perfusion culture was performed using a hollow fiber filter with 0.5 μm pores in order to evaluate the effect of the increased pore size on FVIII:C recovery yield. The FVIII:C recovery yield with a 0.5 μm pore size was 53.4% in the first harvest, 20.1% in the second harvest, and 17.8% in the third harvest, (Fig. 3.3B). FVIII:C recovery yields from the same perfusion day in the ATF perfusion using a hollow fiber membrane filter

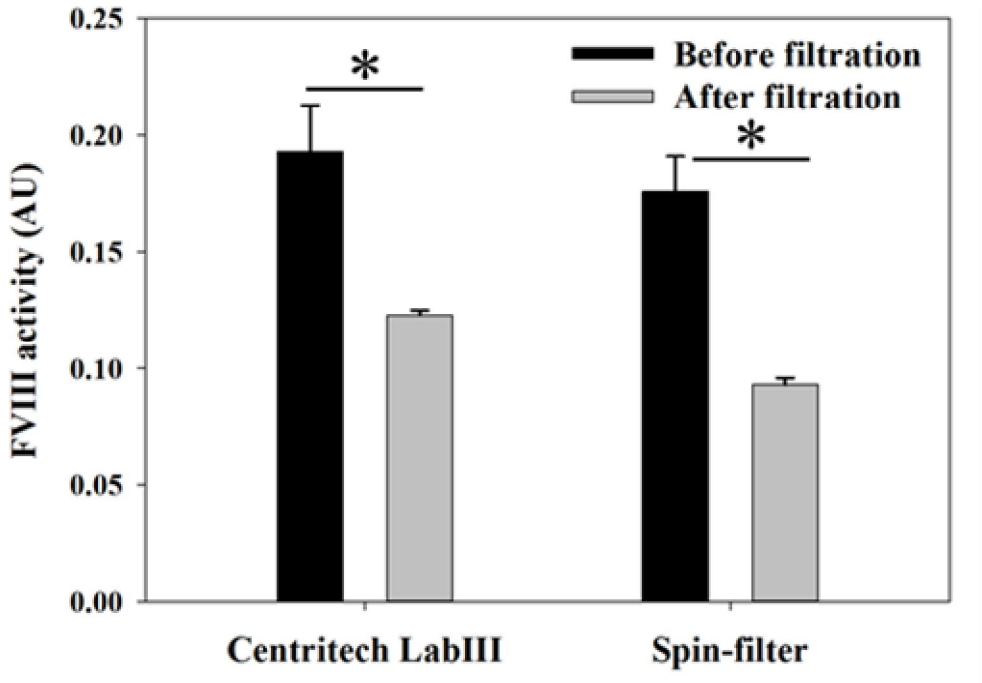


Fig. 3.2 Comparison of FVIII:C between samples before and after dead-end filtration. AU indicates arbitrary unit. Error bars represent standard deviations with n=3. *$P < 0.05$ (two-tailed Student's t-test).

with 0.2 μm pores gave 57.4%, 21.1%, and 16.9%, respectively, as shown in Fig. 3.3A.

The FVIII:C recovery yield of both perfusion cultures was very similar. In addition, a similar decreasing trend in FVIII:C recovery yield over the culture time was observed in both perfusion cultures. Consequently, it is obvious that increasing the pore size of the hollow fiber filter in the ATF perfusion culture is not an exact solution for increasing FVIII:C recovery yield.

3.7 Effect of TMP on FVIII:C recovery yield, FVIII:Ag yield, and vWF:Ag yield

A controlled TMP experiment with a hollow fiber filter was performed in order to evaluate the increased TMP on the FVIII:C recovery yield, FVIII:Ag yield, and vWF:Ag yield. The yields after hollow fiber filtration under controlled TMP are shown in Fig. 3.4. FVIII:C recovery yield, FVIII:Ag yield, and vWF:Ag yield were 93.7%, 91.9% and 89.2%, respectively, on applying TMP of less than 10 psi (the average TMP was 2.83 psi).

However, these yields were rather significantly decreased when a TMP of more than 10 psi was applied. At 10 psi TMP, FVIII:C recovery yield, FVIII:Ag yield, and vWF:Ag yield decreased to 34.6%, 33.6%, and 35.9%, respectively. At above 10 psi, data showed that these yields, FVIII:C recovery yield, FVIII:Ag yield, and vWF:Ag yield, decreased to between 16~20%. These results suggest that the control of TMP under 10 psi can be a good way of enhancing FVIII:C by reducing the retention of FVIII:Ag in an ATF perfusion culture. However, further studies are needed to elucidate the mechanism by which the FVIII yield is decreased under an increased TMP of more than 10 psi.

In addition to fouling of the hollow fiber filter, another important factor to be considered in this study is the multimers of vWF co-expressed with FVIII.

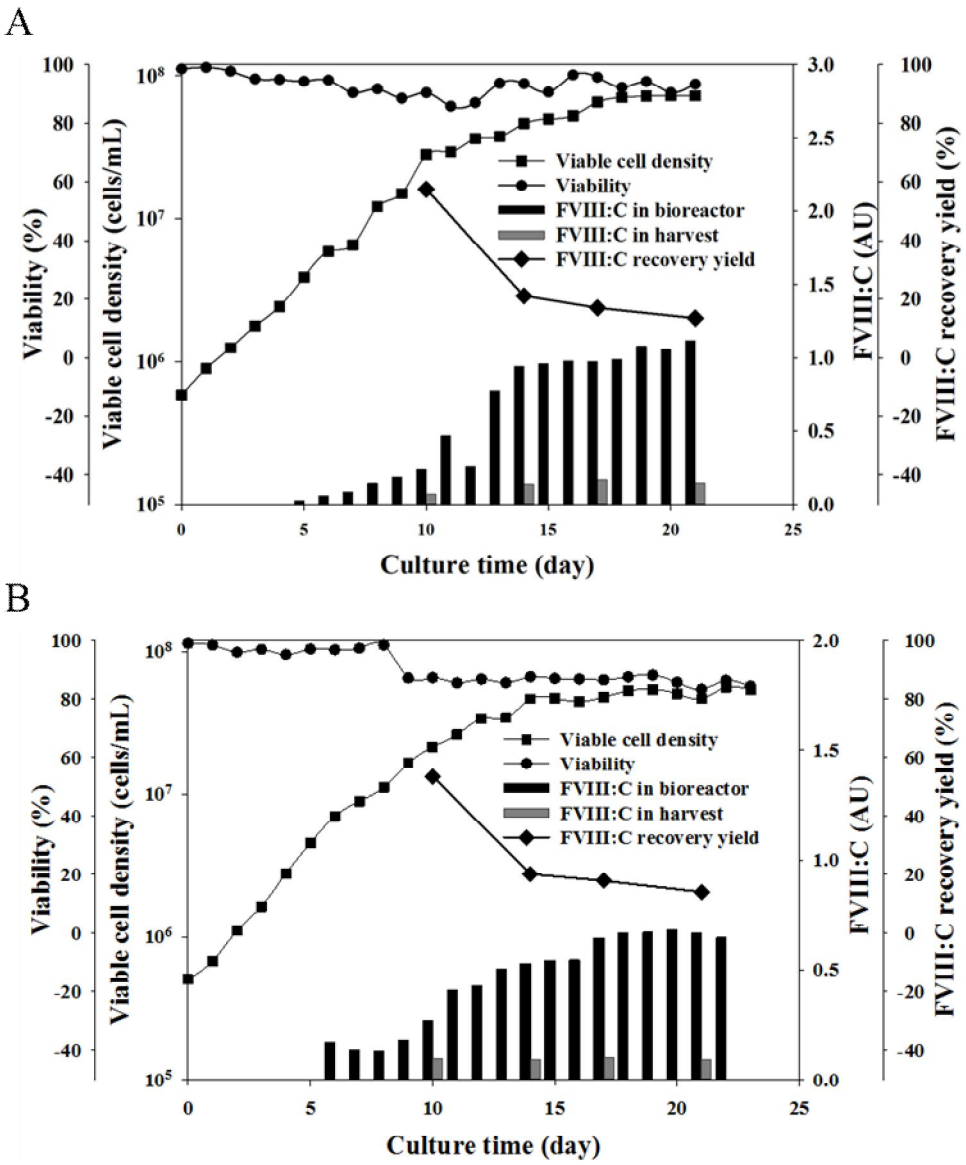


Fig. 3.3 Cell growth and FVIII production profile in an ATF perfusion using a hollow fiber filter with 0.2 μm pores (A) and 0.5 μm pores (B). n=1 for each experiments. AU indicates arbitrary unit.

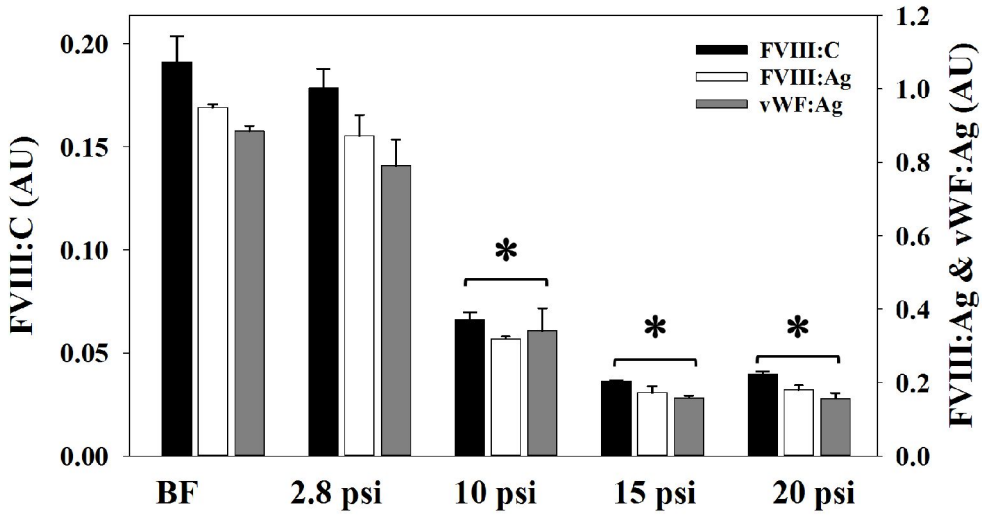


Fig. 3.4 FVIII:C, FVIII:Ag concentration and vWF:Ag concentration after hollow fiber filtration under controlled transmembrane pressure. The measured transmembrane pressure is indicated in the X-axis. BF means before filtration. AU indicates arbitrary unit. Psig is the abbreviation of pounds per square inch gage. Error bars represent standard deviations with n=3. *$P < 0.001$ (two-tailed Student's t-test)

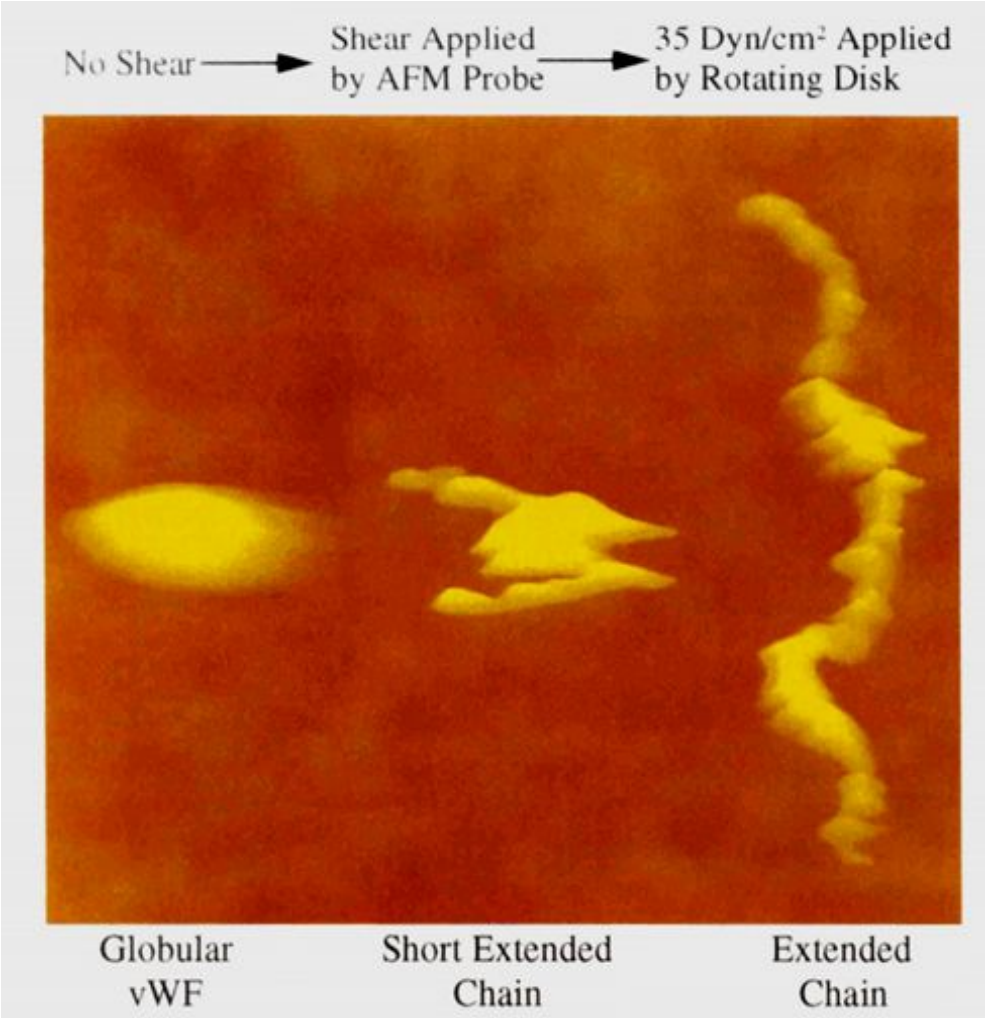


Fig. 3.5 Schematic model using actual AFM images of vWF illustrating shear-induced conformational changes in vWF under aqueous conditions Under negligible shear, vwF has a globular conformation. After exposure to a shear stress field of 35 ± 3.5 dyne/cm^2, vWF is observed in extended conformation with molecular length ranging from 146 to 774nm. AFM; atomic force microscopy (Siediecki *et. al.*, 1996)

As shown in Fig. 3.5, vWF has a long extended chain conformation after exposure a shear stress. It has been reported that the hydrodynamic radius of vWF multimer can be increased up to 466 nm under shear stress (Hoyer and Shainoff, 1980; Shankaran *et al.*, 2003). Also, it has been reported that the fully stretched vWF multimer showed an end-to-end length ranging from 2,300~2,800 nm (Seyfried *et al.*, 2010).

Thus, it can be hypothesized that the co-expressed vWF in this study may partly contribute to the retention of FVIII:Ag due to filtration. However, exact details regarding the effect of vWF multimer on the FVIII yield in ATF perfusion have not yet been reported upon. The current experimental results do not enable further conclusions to be drawn to prove our hypothesis. Thus, further studies are needed in order to better understand the relationship between vWF multimer and the retention of FVIII:Ag.

3.8 Conclusions

In this study, it was reported the observation that FVIII:C recovery yield in an ATF perfusion culture was considerably lower, compared with two other perfusion systems such as a spin-filter and Centritech Lab III centrifuge. The results of this study successfully demonstrated that the low FVIII:C recovery yield in an ATF perfusion culture was not due to the inactivation or surface adsorption of FVIII molecules, but due to the retention of FVIII molecules by hollow fiber filtration.

Controlled TMP below 10 psi resulted in remarkable enhancement of FVIII yield in hollow fiber filtration. These observations suggest that TMP across a hollow fiber filter is a key factor in increasing the product yield of FVIII in an ATF perfusion culture.

However, the FVIII yield was rather decreased under an increased TMP of more than 10 psi. It was also suggested that the reduction of the product yield of FVIII above 10

psi might be due to the conformational change of vWF molecule under shear stress.

Although further studies are needed to develop a precise control method for TMP and to determine an optimal TMP, the findings of this study can be applied to improving the product yield of other target proteins in an ATF perfusion culture.

Chapter IV

Development of a modified harvest system

4.1 Theoretical background

Filtration is a pressure-driven membrane process by which target proteins (solutes) are separated from the supernatant (solvent). In case of a pure solvent flowing under a laminar flow condition, the relationship between the permeate flux and the transmembrane pressure can be described by the following Kozeny-Carman equation (Carman, 1938):

$$J = \frac{\Delta P}{\mu R_m} \qquad (4\text{-}1)$$

where J is the flux (volumetric rate per unit area), ΔP is the transmembrane pressure, μ is the solvent viscosity, and R_m is the membrane resistance.

The filtration process in the presence of a solute is described by the following equation (Bowen and Jenner, 1995):

$$J = \frac{\Delta P - \Delta \pi}{\mu (R_m + R_s)} \qquad (4\text{-}2)$$

where $\Delta \pi$ is the difference in osmotic pressure across membrane and R_s is the total deposition of solute onto the membrane surface. The $R_m + R_s$ represents the total resistance.

According to these equations (4-1 and 4-2), the permeate flux is directly proportional to the TMP when other parameters are the same. It was reported that the yield of target proteins is affected by the TMP (Ahmad, 2012). Hirasaki *et al.* (Hirasaki *et al.*, 1995) also showed that the permeability of two kinds of DNA with different molecular weights, λDNA (MW = $3X10^7$) and Bacillus sp. DNA (average MW = $1X10^8$) is increased when the TMP increased. In chapter III of this study, it was demonstrated that the controlled TMP below 10psi resulted in remarkable enhancement of FVIII yield in hollow fiber filtration. And it was suggested that the TMP across hollow fiber filter is a key factor

to increase the product yield of FVIII in ATF perfusion culture.

The aim of this chapter is to develop a device in order to increase the TMP across hollow fiber filter. The transmembrane pressure across hollow fiber filter of ATF system is calculated as using the following formula:

$$TMP = \frac{P_{in} + P_{out}}{2} - P_{perm} \qquad (4\text{-}3)$$

where P_{in} is the inlet pressure at the bioreactor connection port, P_{out} is the outlet pressure at the second chamber of a diaphragm pump, and P_{perm} is the permeate pressure at the harvest port.

According to the equation 4-3, there are two methods to increase the TMP. One is to increase P_{in} and P_{out}. Unfortunately, P_{in} and P_{out} is a function of the diaphragm pump speed which directly affects cell viability and cell growth negatively. P_{in} and P_{out} could be controlled by squeeze the connection tubing between the bioreactor and the bioreactor connection port of ATF. However, this method results in the increase of shear stress which also might affect cell viability negatively. Thus, there are some limitations to increase the TMP by increasing P_{in} or P_{out}. The other is to decrease P_{perm}. However, it is difficult to decrease P_{perm} in the peristaltic harvest system as described in Chapter IV. 4.5. Thus, it was strongly required to develop an alternating harvest system eliminating the need of a peristaltic pump. In latter part of this chapter, the design and the principle of the novel harvest system was described. The application of the system was described in Chapter V.

4.2 Peristaltic pump harvest system

Peristaltic pumps have been widely used for many years in scientific, medical and industrial applications whereby a fluid is delivered through a single compressible tube

at a controlled rate. The pumping principle is based on the peristaltic motion of a peristaltic pump. The Webster's definition of peristalsis is "the successive waves of involuntary contraction passing along the walls of a hollow muscular structure (as the esophagus or intestine) and forcing the contents onward" Peristalsis is a series of wave-like muscle contractions that moves food to different processing stations in the digestive tract.

Peristaltic pump is composed of three parts: drive, pump head and tube. Typically, the pump head of peristaltic pump has a rotor with several rollers. As the rotor turns, the flexible tubing is squeezed at each roller location. And then the flexible tubing is released. The alternating pattern of squeezing and releasing the flexible tubing moves the fluid through the peristaltic pump. The part of tubing under compression by roller of a peristaltic pump is always in a closed state.

Peristaltic pumps transfer fluids successfully in industries such as food processing, pharmaceutical manufacturing, and chemical processing, as well as in laboratory research, agriculture, and water treatment. The most important advantage of peristaltic pumps is their use of tubing as the pump chamber. The fluid is inside the tubing and does not contaminate the pump, nor does the tubing contaminate the fluid. The biggest advantage of the use of peristaltic pumps is to maintain fluid aseptic condition. Fluid does not contact any part of the pump except inside the tubing. In the whole process of the pharmaceutical industry, the aseptic condition is a basic requirement of the general. The peristaltic pump with high flow precision can satisfy the transmission of various quantitative pharmaceutical process requirements. In the process of production of raw medicine synthesis, extraction and culture, peristaltic pumps are used to add all kinds of acids, solvents, alkalis, nutrient solution, buffer solution and raw materials and are used to sampling and collection.

A typical application is the application of peristaltic pump in bioreactor for

microbial and mammalian cell culture. Peristaltic pumps are used to add cell culture medium, feed solution, and base. Peristaltic pumps are also used to sampling. Especially in perfusion culture, two peristaltic pumps are mainly and generally used for the addition of fresh medium and/or the withdrawal of spent medium, i.e. perfusion rate control. Peristaltic pumping has been described for perfusion rate control in the application of the ATF system to perfusion culture (Clincke *et al.*, 2013).

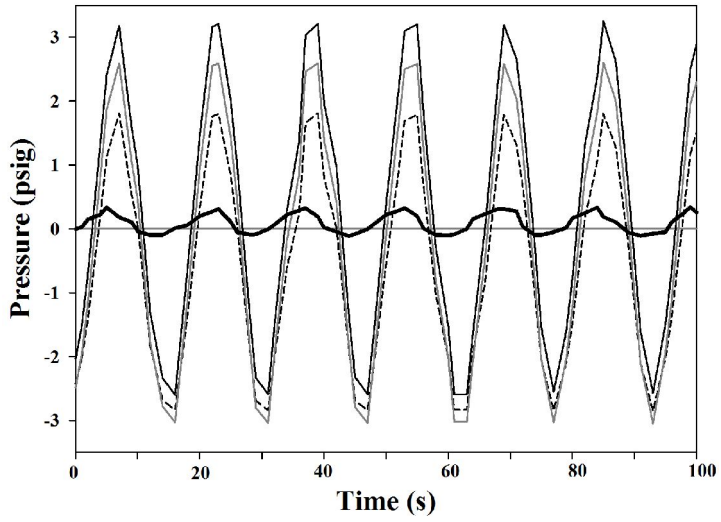
4.3 TMP in the peristaltic harvest system

In chapter III, it was demonstrated that the controlled TMP below 10psi resulted in remarkable enhancement of FVIII yield in hollow fiber filtration. And it was suggested that the TMP across hollow fiber filter is a key factor to increase the product yield of FVIII in ATF perfusion culture. Therefore, it was necessary that the transmembrane pressure across hollow fiber filter should be measured during the operation of ATF system with a peristaltic pump harvest system.

Fig. 4.1 shows the time profile of transmembrane pressure during on time and off time in the peristaltic harvest system. The TMP profile shows a somewhat different pattern over cycles. It was thought that this result might be due to the resolution problem of the recording software used in this study. The minimum data collection period of the recording software is 1 second. '1 second' does not seem to be enough time to collect enough data to show a precise TMP profile. If higher-resolution recording software were used to collect data, we believe, more similar and repeating TMP profiles could be obtained.

As shown in Fig. 4.1, it is obvious that there was a marginal difference between the time profiles of TMP. Even in the peristaltic pump on time, the TMP did not increased unexpectedly. The average TMP and the average peak TMP in the peristaltic pump harvest system was 0.13 ± 0.04 psig and 0.16 ± 0.005 psig, respectively.

A



B

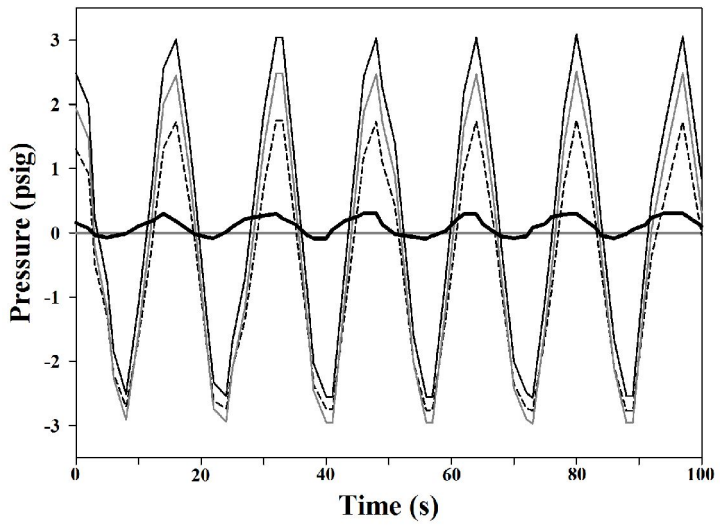


Fig. 4.1 Time profile of TMP during the Peristaltic pump on-time (A) and off-time (B) Transmembrane pressure (thick solid line), inlet pressure (solid line), outlet pressure (gray line) and permeate pressure (dashed line). Psig is the abbreviation of pounds per square inch gage.

4.4 Effect of the pump speed on TMP in the peristaltic harvest system

The time profile of TMP according to the peristaltic pump speed was measured because the increase of the pump speed can affect the permeate pressure by increasing the amount of filtrate flowed into a harvest bag by the suction of the peristaltic pump.

As shown in Fig. 4.2, the measured peak of TMP was 0.157 psig for 5 rpm, 0.143 psig for 15 rpm, 0.158 psig for 30 rpm, and 0.160 psig for 50 rpm, on average. The measured peak of permeate pressure was 2.17 psig for 5 rpm, 2.16 psig for 15 rpm, 2.18 psig for 30 rpm, and 2.19 psig for 50 rpm on average. The pump speed of Fig. 4.1 is 14.5 rpm.

As a result, increasing the suction speed of the peristaltic pump could not increase the TMP. Since the increase of the permeate flux is proportional to the increase of TMP, an increase of peristaltic pump speed (i.e. the suction speed) would not increase the permeate flux. In conclusion, the FVIII yield could not be increased by increasing the peristaltic pump speed.

4.5 Importance of the occlusion of the silicone tubing in the peristaltic pump harvest system

Typically, a peristaltic pump has a rotor with several rollers. The flexible tubing is squeezed at each roller location. As the rotor turns, the part of tubing under compression by roller of a peristaltic pump is always in a closed state. In other words, the harvest tube is always occluded by a peristaltic pump. In most cases, even though in ATF perfusion culture, this occluded state is not an important consideration, since the size of the secreted proteins is small enough to pass through the hollow fiber filter in the peristaltic harvest system as reported in Clincke *et al.* (2011).

However, if the size of the target protein is as large as the pore size of hollow

fiber filter, the situation is totally different. In high cell density perfusion culture, cells as well as cell debris could block membrane pores partially. And although the less formation of biofilm is the main advantage of ATF system, biofilm forms in some degree at high cell density culture. Due to these two reasons, the effective pore size of hollow fiber filter could be decreased. Thus, if the molecular size of the target proteins is large enough to have difficulties to pass through the pore of hollow fiber filter, the occlusion of the harvest line could be an important issue.

As described previous section, the increase of TMP is a little difficult by increasing P_{in} or P_{out}. Moreover, the increase of TMP by decreasing P_{perm} is also highly difficult due to the lasting occlusion of the harvest line in the peristaltic pump harvest system. This is the reason to develop a new harvest system as an alternating of a peristaltic pump in ATF perfusion culture.

4.6 Design of the modified harvest system

As described in 4.1 of Chapter IV, the TMP is highly important in the FVIII yield in ATF perfusion culture. It can be assumed that if the TMP during the pressure cycle can be increased and maintained positive value, the FVIII yield could be increased. Based on this assumption, the modified harvest system composed of a check valve, a pinch valve (Fig. 4.3-7) and a timer was developed.

In the system, the critical point is to minimize the permeate pressure or backpressure on the filtrate side and to maintain the positive TMP during the pressure cycle. To achieve these purpose, three key components such as a check valve, a pinch valve and a timer were applied. The characteristic functions of each parts were described in detail below.

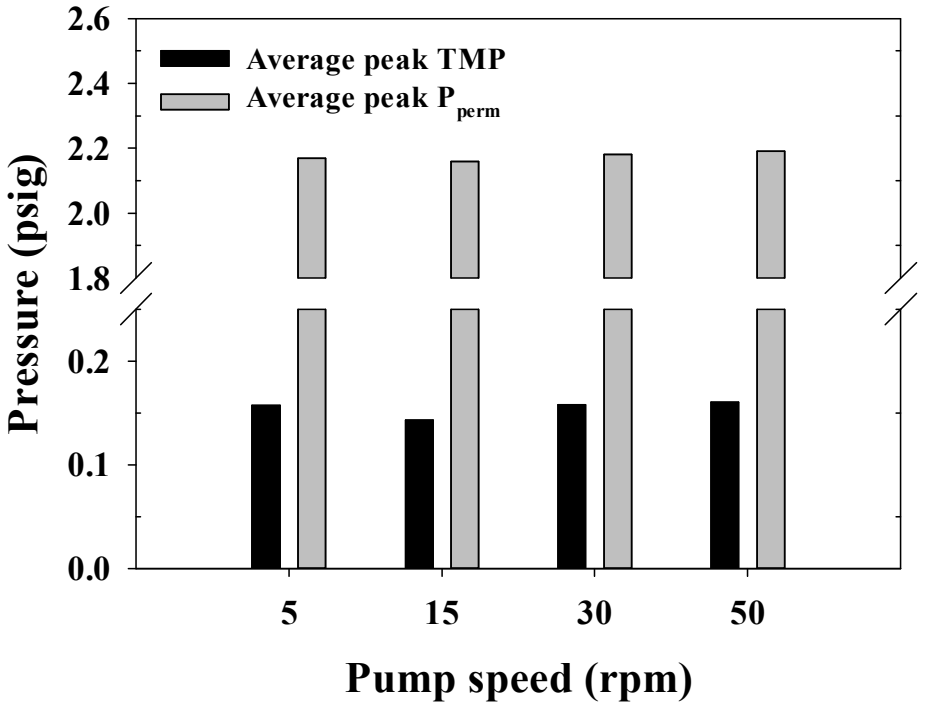


Fig. 4.2 Measurement of average peak TMP and average peak P_{perm}. TMP: transmembrane pressure, Pperm: permeate pressure. Psig is the abbreviation of pounds per square inch gage.

4.6.1 Check valve

The check valve (code no. 418, Kartell, Italy) as shown in Fig. 4.3 is a non-return valve or one-way valve, which allows the flow of fluid in only one direction. As shown in Fig. 4.5-7, the check valve was connected to the harvest port of ATF-4 system (Refine Technology, USA) through a 3/16-inch internal diameter silicone tubing (Saint-Gobain, France).

During the pressure cycle, the check valve is always open and allows the filtered supernatant to flow smoothly toward the harvest bag. During the exhaust cycle, the check valve is closed, blocking the backward flow of the filtered supernatant into inner side of hollow fiber filter (Fig 4.4).

This function of the check valve is essential for ATF action. If the backward flow of the filtered supernatant is not prevented by the check valve when the harvest tubing is opened, the filtered supernatant can flow back into inner side of the hollow fiber filter during the exhaust cycle, and the cell-containing supernatant will not be properly driven from the bioreactor vessel to the second chamber of the diaphragm pump.

As a result, a certain amount of the filtered supernatant can flow back and forward between the inner and outer sides of the hollow fiber filter and the cell containing supernatant can oscillate between bioreactor and the second chamber of the diaphragm pump. Consequently, perfusion culture cannot be properly performed.

The use of a check valve enables the minimization of permeate pressure in ATF perfusion. This characteristic function of a check valve was described in detail in 4.9 and 4.10 of this chapter.

4.6.2 Pinch valve

A model RZ-98305-11 pinch valve (Cole-Parmer, USA) was installed after the

check valve to open and close silicone tubing between the check valve and the harvest bag (Fig. 4.3-7). If only the check valve is used for harvest, all the culture supernatant will be continuously filtered out and the vessel will eventually empty. The pinch valve with a timer also has an important role in weight-based perfusion rate control with a load cell installed under the bioreactor vessel. Since the pinch valve opens and closes the silicone tube in a timed fashion, the perfusion rate can be properly controlled.

4.6.3 Timer

In the modified harvest system, one of the crucial aspects is the use of a timer (LE7M, Autronics, USA). The purpose of the use of the timer is to maintain the open state of the harvest tube during pressure cycle of pinch valve off-time. The timer was connected to the pinch valve and the load cell (Fig. 4.5).

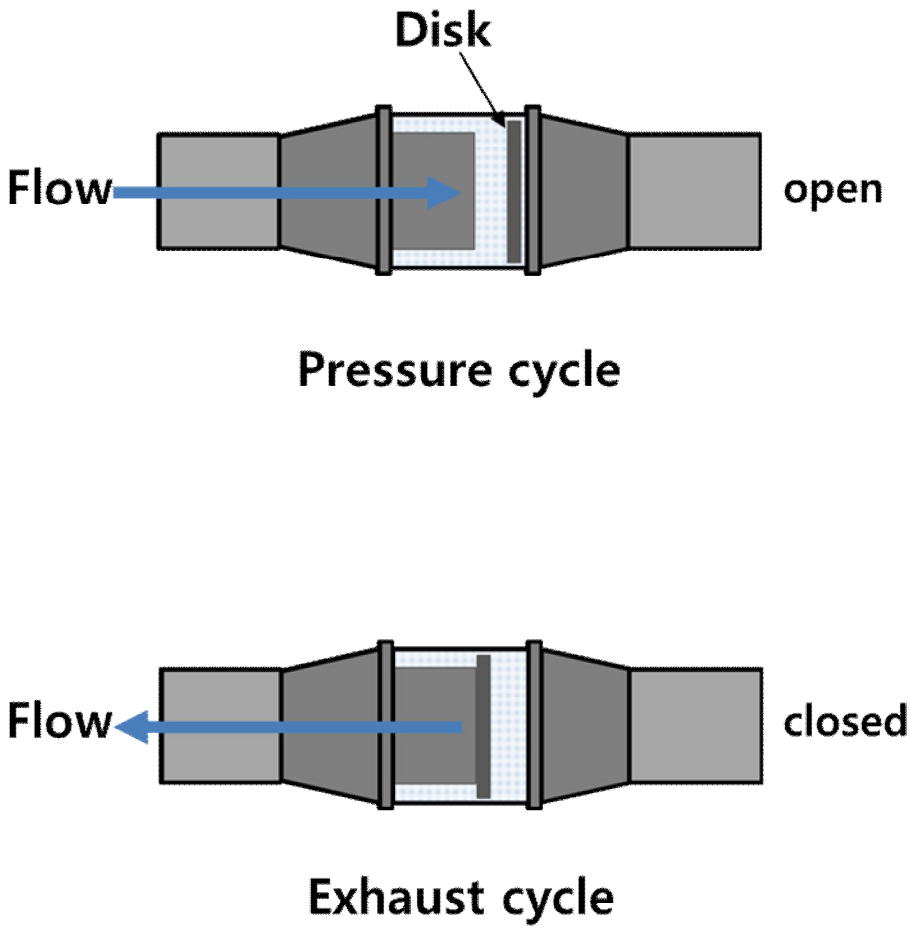
The timer actuates the pinch valve in accordance with the signal from the load cell. If the vessel weight is increased to the set-point, the load cell gives a signal to the timer connected to the pinch valve. The timer allows the pinch valve to open for one minute (pinch valve off-time). After one minute, the vessel weight is decreased below the set-point and the controller confers the close signal to the pinch valve (pinch valve on-time).

If the pinch valve is directly operated without the timer, the load cell sends on-and-off signals to the pinch valve repeatedly around the vessel weight set-point. In this situation, the harvest tube is quite frequently in the closed state during both pressure cycle and exhaust cycle.

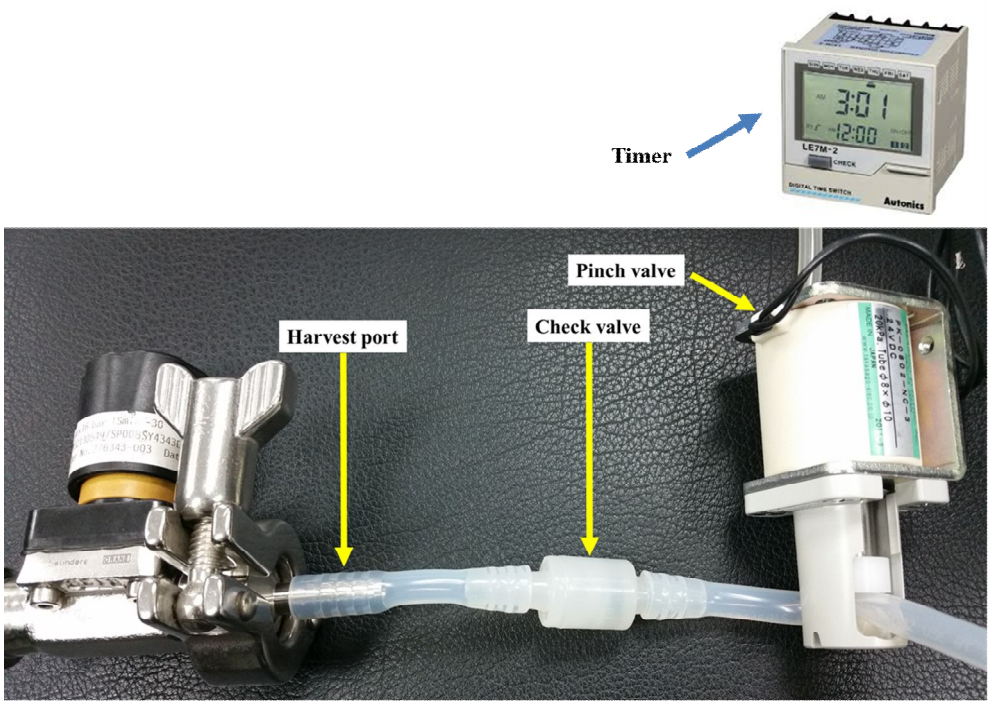
A

B

4.3 A check valve (A) and a pinch valve (B) The check valve (code no. 418, Kartell, Italy) as shown in A is a non-return valve or one-way valve, which allows the flow of fluid in only one direction. The pinch valve (model RZ-98305-11, Cole-Parmer, USA) as shown in B is a type of control valve which uses a pinching effect to obstruct fluid flow.



4.4 Schematic representation of the operation of a check valve The check valve always open and allows the filtered supernatant to flow smoothly toward the harvest bag during the pressure cycle. During the exhaust cycle, the check valve is closed, blocking the backward flow of the filtered supernatant into inner side of hollow fiber filter.



4.5 Connection of a check valve, a pinch valve and a timer

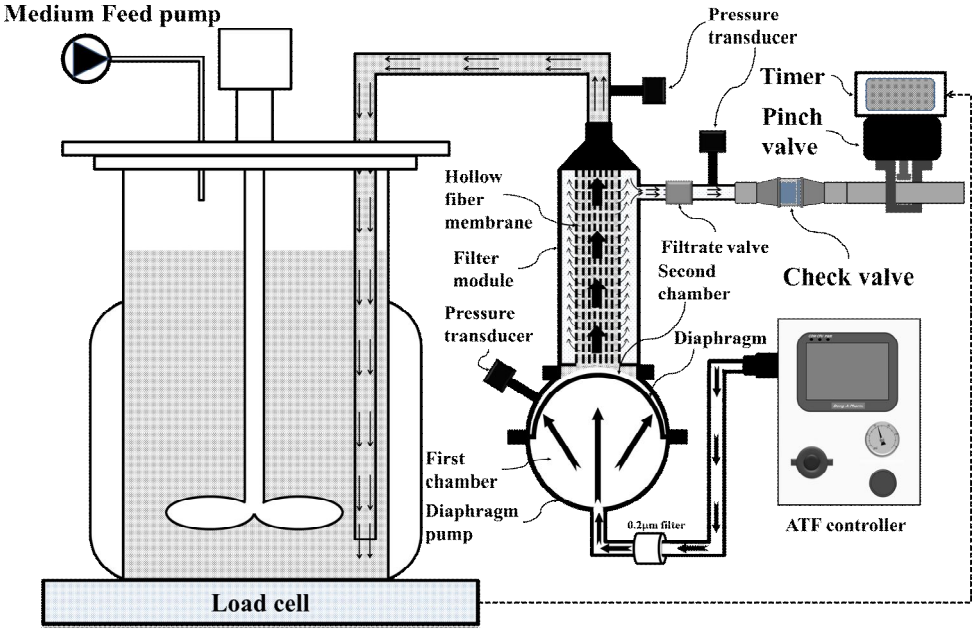


Fig. 4.6 Schematic view of ATF perfusion system with a modified harvest system at the pressure cycle

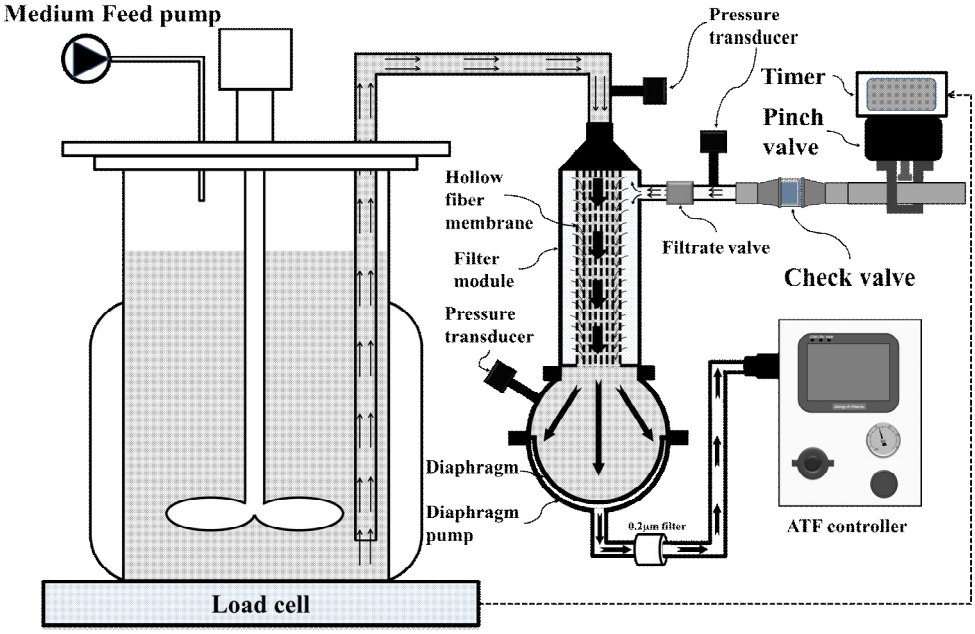


Fig. 4.7 Schematic view of ATF perfusion system with a modified harvest system at the exhaust cycle

4.7 Determination of the pinch valve off-time

The pinch valve off-time is directly related to the control of the perfusion rate in ATF perfusion culture. The pinch valve off-time is directly controlled by the timer connected to the pinch valve and the load cell. The increase of the pinch valve off-time during the pressure time means the increase of the amount of the filtrate flowed into a harvest bag (i.e. the harvest volume). The increase of the harvest volume results in the rapid change of the bioreactor volume which can affect DO, pH, temperature and cells.

First of all, the flow rate of the filtrate into a harvest bag was measured in order to optimize the duration time of the pinch valve off-time. The flow rate over time was shown in Table 4.1. The measured average flow rate per minute was approximately 220mL/min. The change of the bioreactor volume for 1 minute was 7.4% of the working volume. This amount of volume change did not result in a fluctuation of DO, pH or temperature and did not affect cell viability or cell growth. Therefore, 1 minute was chosen as the duration of the pinch valve off-time.

Based on the data of Table 4.1, the cycle was composed of a 1 min of the pinch valve off-time and a 79 min of the pinch valve on-time. In total, 18 cycles per day were conducted at1vvd. The theoretical volume of the filtrate per day was 3L. The actual volume of the filtrate per day was 2.98L. From these data, it was concluded that the perfusion rate was successfully controlled by adjusting the on-and off-time of the pinch valve.

4.8 TMP in the modified harvest system

The TMP across hollow fiber filter was measured during the operation of ATF system with the modified harvest system. Fig. 4.8 and Fig. 4.9 shows the time profile of TMP during on-and off-time of the modified harvest system. The TMP profile showed also a somewhat different pattern over cycles as in the case of the peristaltic pump harvest

Table 4.1 Determination of the pinch valve off-time

Time (min)	Cumulative filtrate volume (mL)	Difference (mL)
1	248.7 ± 3.2	-
2	472.7 ± 3.1	224.0
3	690.3 ± 7.5	217.7
4	921.3 ± 3.2	231.0
5	1132.7 ± 5.5	211.3
Average	248.7 ± 3.2	221

Mean± SD (n=2)

system. It was also thought that this result might be due to the resolution problem of the recording software used in this study.

As shown in Fig. 4.8, the time profile of TMP during the pinch valve on-time was quite similar to that during the peristaltic pump on- and off-time according to expectation. However, the time profile of TMP during the pinch valve off-time was totally different from that of the peristaltic pump harvest system (Fig. 4.9). The permeate pressure was dramatically decreased during the pressure cycle. The TMP was also increased and maintained a positive value during the pressure cycle. These results demonstrated that the permeate pressure was successfully decreased by the use of the pinch valve and the check valve. From these data, it can be concluded that the modified harvest system can achieve the enhanced FVIII yield through the TMP increase.

4.9 Comparison of the TMP profiles of the peristaltic pump harvest system and of the modified harvest system

The time profile of TMP variation in both the peristaltic pump system and the modified harvest system was compared, and is depicted in Fig. 4.10. Since the yield of target proteins is affected by the TMP during harvest (Ahmad, 2012; Hirasaki *et al.*, 1995; Salgin and Salgin, 2007), the TMP in the pinch valve off-time of the modified harvest system was compared with that in the operation time of the peristaltic pump.

In the peristaltic pump harvest system, TMP was rapidly increased with the start of pressure cycle and had its maximum value in the last time of the pressure cycle. During the exhaust cycle, the TMP was also rapidly decreased until back flushing by a negative TMP occurred (Shevitz, 2003). However, the time profile of TMP variation in the pinch valve off-time was significantly different from the peristaltic pump. The TMP was maintained positive, regardless of the pressure or the exhaust cycle. TMP fluctuated between 0.22 and

0.31 psig. The average TMP and the average peak TMP of three pressure cycles was 0.13 ± 0.04 psig and 0.16 ± 0.005 psig, respectively, in the peristaltic pump harvest system and 0.25 ± 0.03 psig and 0.31 ± 0.003 psig, respectively, in the modified harvest system (Fig. 4.9). The modified system achieved an 89% higher average TMP and a 96% higher peak TMP in the pressure cycle ($P < 0.001$, two-tailed Student's *t*-test). As a result, the enhancement of the recovery yield of FVIII:C resulted from the increase of TMP.

As shown in Fig. 4.11, there were significant decreases in the peak permeate pressure to 44.8%, peak outlet pressure to 37.5% and peak inlet pressure to 61.5% on average in the pressure cycle compared with the peristaltic pump harvest system. In addition, the duration time of the pressure cycle was decreased to 5 seconds from 8.7 seconds in the peristaltic pump harvest system (Fig. 4.10). The duration time of exhaust cycle was 7 seconds in both harvest systems. This result means that it took less time to drive the water from the second chamber of a diaphragm pump to the bioreactor vessel.

These results were probably due to the function of the check valve. When the check valve and the pinch valve were used instead of the peristaltic pump, there was no blockage of the harvest tube during the pressure cycle in the pinch valve off-time. In other words, there was little permeate back pressure due to the occlusion of harvest tube in the pressure time. This probably led to the reduction of permeate pressure and a resultant increase of permeate flux, which in turn resulted in decreased outlet flux. This caused a decrease of outlet pressure. Also, the decreases of both outlet and permeate pressure were accompanied by the decrease of inlet pressure. Since the flow-disturbing pressure, such as the outlet and permeate pressure, was decreased, the pressure cycle time was decreased under a constant driving force by a diaphragm pump in the ATF system. Consequently, it is obvious that the results as shown in Fig. 4.10 and Fig. 4.11 were due to this characteristic function of the check valve.

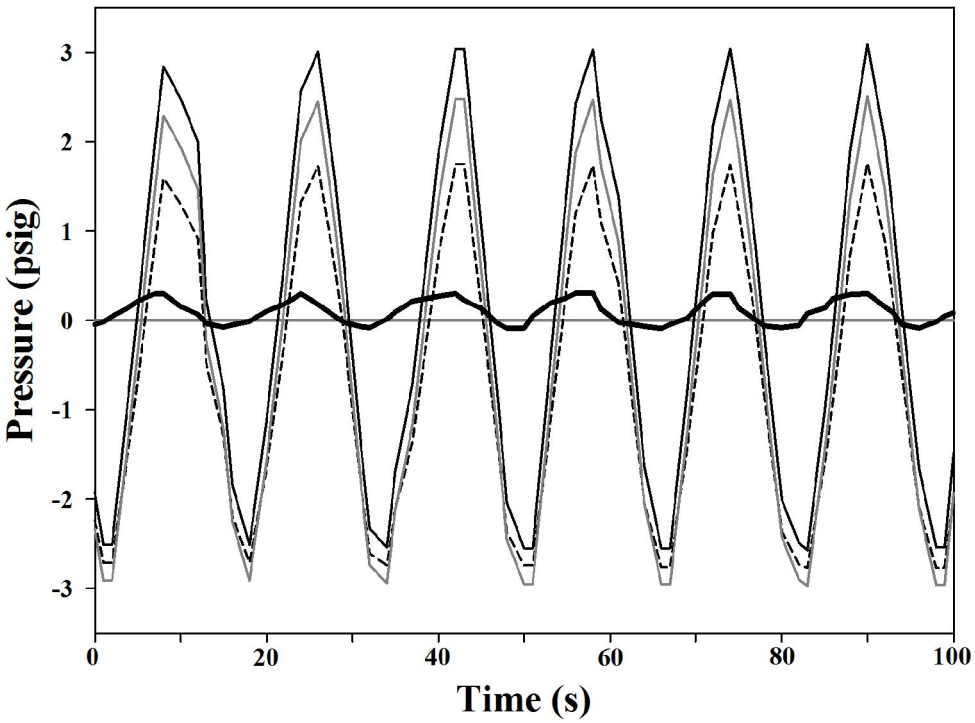


Fig. 4.8 Time profile of TMP during the pinch valve on-time in the modified harvest system. Transmembrane pressure (thick solid line), inlet pressure (solid line), outlet pressure (gray line) and permeate pressure (dashed line). Psig is the abbreviation of pounds per square inch gage.

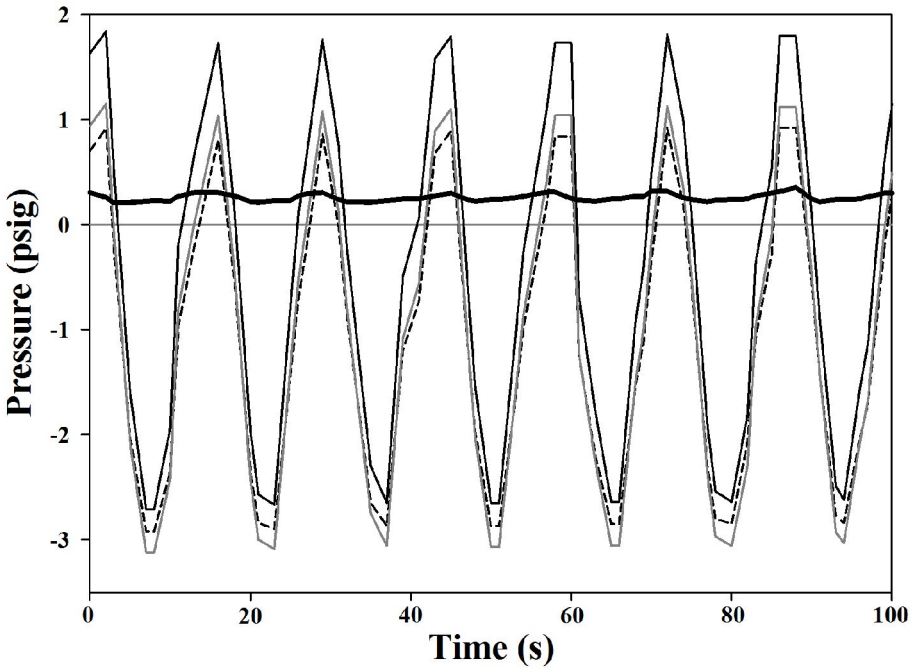


Fig. 4.9 Time profile of TMP during the pinch valve off-time in the modified harvest system. Transmembrane pressure (thick solid line), inlet pressure (solid line), outlet pressure (gray line) and permeate pressure (dashed line). Psig is the abbreviation of pounds per square inch gage.

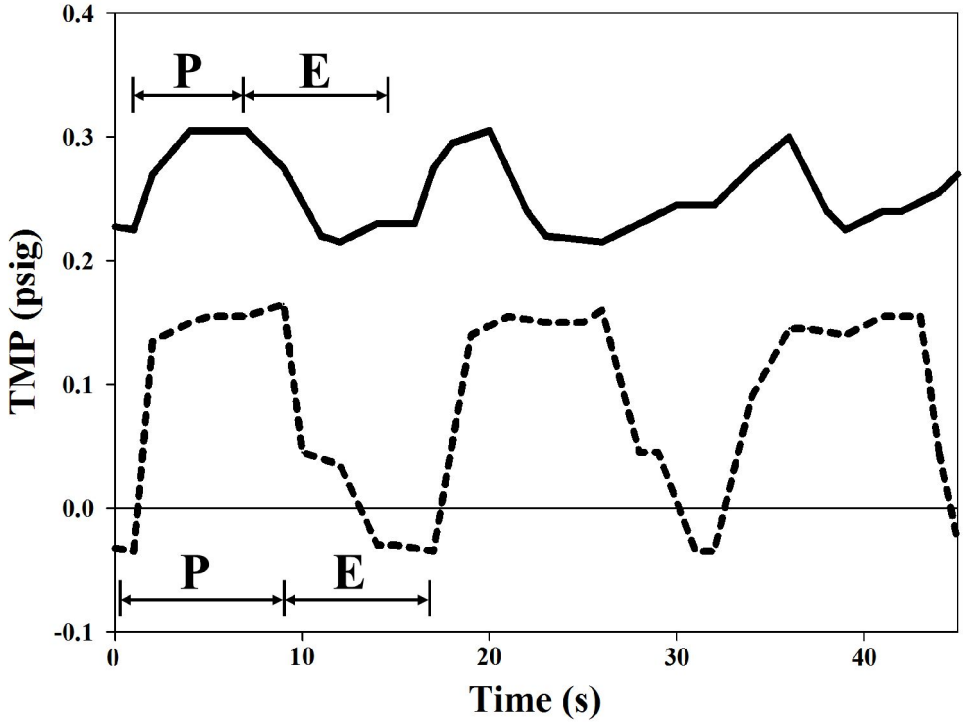


Fig. 4.10 Results of TMP measurement during pinch valve off-time (thick solid line) and peristaltic pump on-time (thick dashed line). P: pressure cycle. E: exhaust cycle. Psig is the abbreviation of pounds per square inch gage. Error bars represent standard deviations with n=3. *$P < 0.001$ (two-tailed Student's *t*-test).

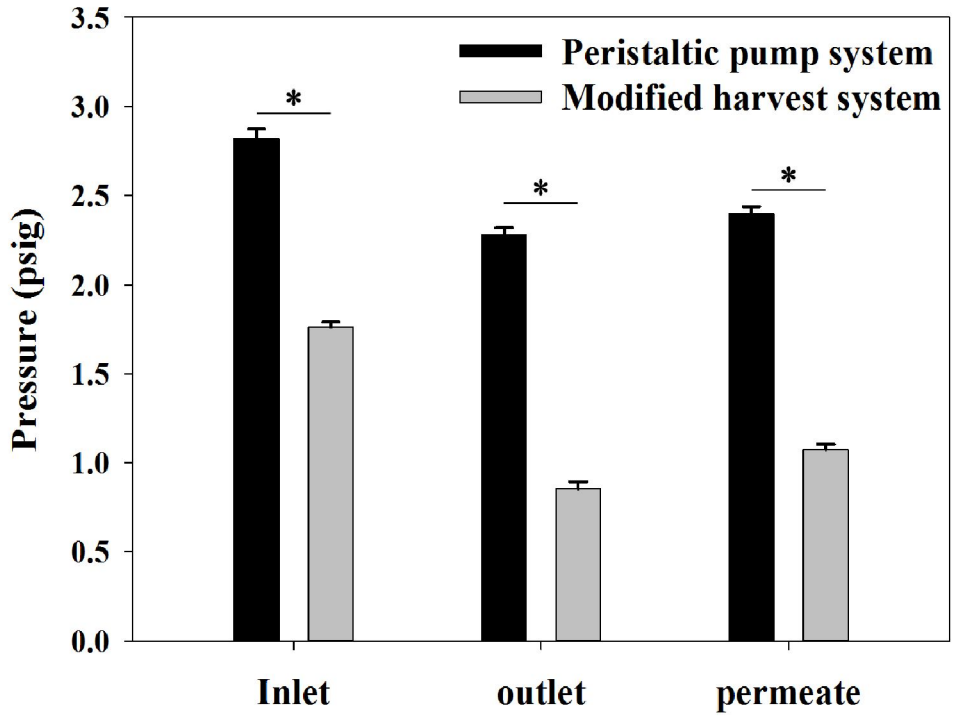


Fig. 4.11 Comparison of peak pressures. P: pressure cycle. Inlet: inlet pressure. outlet: outlet pressure. permeate: permeate pressure. Psig is the abbreviation of pounds per square inch gage. E: exhaust cycle. Error bars represent standard deviations with n=3. *$P < 0.001$ (two-tailed Student's *t*-test).

4.10 Conclusions

In Chapter IV, it was described the development and experimental verification of a modified harvest system to enhance Factor VIII (FVIII) yield in an alternating tangential flow (ATF) perfusion culture. The modified harvest system was composed of a check valve, pinch valve and timer. The main innovation of the system is the use of check and pinch valves, eliminating the need of a peristaltic pump for harvest. The performance of the modified harvest system was compared with a peristaltic pump in terms of TMP across hollow fiber membrane. The system was successfully applied to ATF perfusion culture.

As expected, the permeate pressure was decreased by the use of a check valve and a pinch valve. As a result of the decrease of permeate pressure, the modified harvest system achieved the higher TMP compared with a peristaltic pump harvest system.

The increased TMP was due to the characteristic function of a check valve. When the check valve was used instead of the peristaltic pump, there was no blockage of the harvest tube during the pressure cycle in the pinch valve off-time. This resulted in the reduction of permeate pressure. The increase of TMP was due to the reduction of permeate pressure.

In Chapter III, it was shown that TMP across a hollow fiber filter is as key factor in increasing the product yield of FVIII in an ATF perfusion culture. Thus, it was suggested that the modified harvest system developed in this study could be useful to enhance the product yield of FVIII in ATF perfusion culture.

Chapter V

Application of the modified harvest system to ATF perfusion culture

5.1 Cell growth and FVIII production

In Chapter V, the performance of the modified harvest system was compared with that of the peristaltic pump harvest system. Fig. 5.1 shows the results of cell growth and FVIII production in ATF no. 1 and ATF no. 2 as described in Chapter II. Although there was an approximate 2-fold difference in seeding densities, the overall cell growth pattern was comparable in ATF no. 1 and ATF no. 2, except for the late stage of the runs. From cell growth results, the modified harvest system showed the possibility of its application to ATF perfusion culture.

In addition to cell growth, the control of perfusion rate is especially important in perfusion culture. As shown in Fig. 5.1, the perfusion rate was successfully controlled using the modified harvest system composed of a check valve and a pinch valve without a conventional peristaltic pump.

Unlike cell growth, ATF no. 1 and ATF no. 2 showed markedly different profiles of FVIII production. In ATF no. 1, The FVIII:C in bioreactor continuously increased over culture time. In Chapter III, it was demonstrated that the increase of FVIII:C in bioreactor was due to the increase of the retained FVIII:Ag. Contrary to ATF no. 1, there was only a marginal increase in FVIII:C in ATF no. 2 over time. The average FVIII:C in the ATF no. 2 bioreactor was decreased to 67% compared with ATF no. 1. From this result, it is inferred that the retention of FVIII:Ag may be decreased in ATF no. 2 compared with ATF no. 1. This difference of FVIII:C in two harvest systems was further analyzed in the following section.

Despite the low average FVIII:C in the bioreactor, these results indicate the potential of the modified harvest system as an alternating method to replace a peristaltic pump harvest system.

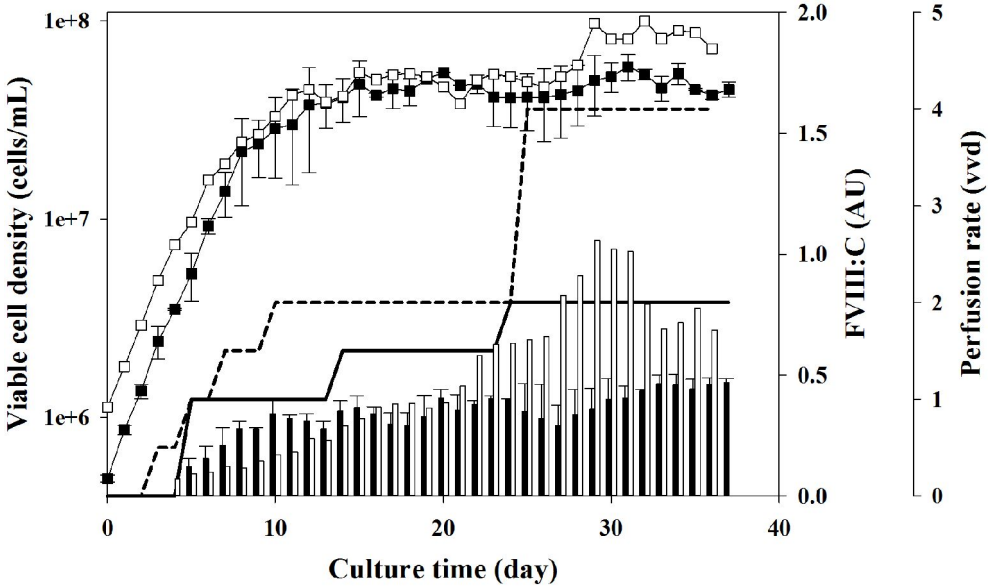


Fig. 5.1 Cell growth and FVIII production profile. Viable cell density of ATF no. 1 run (open square) and ATF no. 2 run (closed square). FVIII:C in bioreactor of ATF no. 1 run (open bar) and ATF no. 2 run (solid bar). Perfusion rate of ATF no. 1 run (dashed line) and ATF no. 2 run (solid line). AU indicates arbitrary unit. Error bars represent standard deviations with n=2.

5.2 Analysis of recovery and product yield

FVIII production profiles were examined in detail to evaluate the effect of the modified harvest system on FVIII:C recovery (Fig. 5.2 and Fig. 5.3). Both ATF no. 1 and ATF no. 2 showed the same decreasing pattern of recovery yield over the perfusion time. However, the final recovery yield was 3.5% in ATF no. 1 and 47.4% in ATF no. 2 (Fig. 5.2). In comparison with ATF no. 1, ATF no. 2 achieved a 13.6-fold increase in the final recovery yield and a 4.3-fold increase in the average recovery yield.

The product yield was also increased in ATF no. 2 compared with ATF no. 1 (Fig. 5.3). In ATF no. 1, the product yield was decreased to 58.7%. In ATF no. 2, the product yield exceeded 85% after perfusion day 10. In comparison with ATF no. 1, ATF no. 2 achieved a 1.47-fold increase in the product yield.

These results indicate that the modified harvest system was more efficient in harvesting the produced FVIII than the peristaltic pump system.

5.3 Analysis of the retention of proteins

The retention of the produced proteins in ATF no. 1 and ATF no. 2 runs was examined (Fig. 5.4). A comparable pattern was observed in the recovery yields of three proteins in ATF no. 2. The recovery yields of FVIII:Ag and vWF:Ag were decreased over the perfusion time as the recovery yield of FVIII:C was decreased. This indicated the retention of FVIII and vWF molecules by the hollow fiber filter in the modified harvest system.

Compared with ATF no. 1 data, the recovery yield of FVIII:Ag of ATF no. 2 was increased 2.4-fold at day 11, 4.5-fold at day 16 and 17.9-fold at day 35, respectively. Furthermore, the recovery yield of vWF:Ag of ATF no. 2 was also increased 2.6-fold at

day 11, 3.2-fold at day 16 and 16.4-fold at day 35, respectively.

Taken together, these results indicate that the retention of proteins by the hollow fiber filter was significantly reduced by the modified harvest system ($P < 0.05$, two-tailed Student's *t*-test), and that the enhancement of the recovery yield of FVIII:C was due to the reduction of retained FVIII:Ag by the hollow fiber filter.

5.4 Conclusions

In this study, the modified harvest system described in Chapter IV was applied in an ATF perfusion culture for FVIII production. The system achieved the comparable cell growth compared with a peristaltic pump harvest system. In addition, the perfusion rate was successfully controlled by combining a check valve and a pinch valve. These results imply that the modified harvest system can be an alternating method to replace a peristaltic pump harvest system in ATF perfusion culture.

As a result of the increased TMP described in Chapter IV, the modified harvest system achieved the enhancement of the recovery and product yield of FVIII:C. This modified harvest system could be beneficial to the enhancement of the yield of other recombinant proteins using an ATF system.

Taken together, the modified harvest system for ATF perfusion culture was successfully applied in a perfusion culture for FVIII production. Moreover, the modified harvest system offers an efficient alternating method for harvesting and provides a promising tool to solve the fouling problem in the filtration-based perfusion system, especially in ATF perfusion culture.

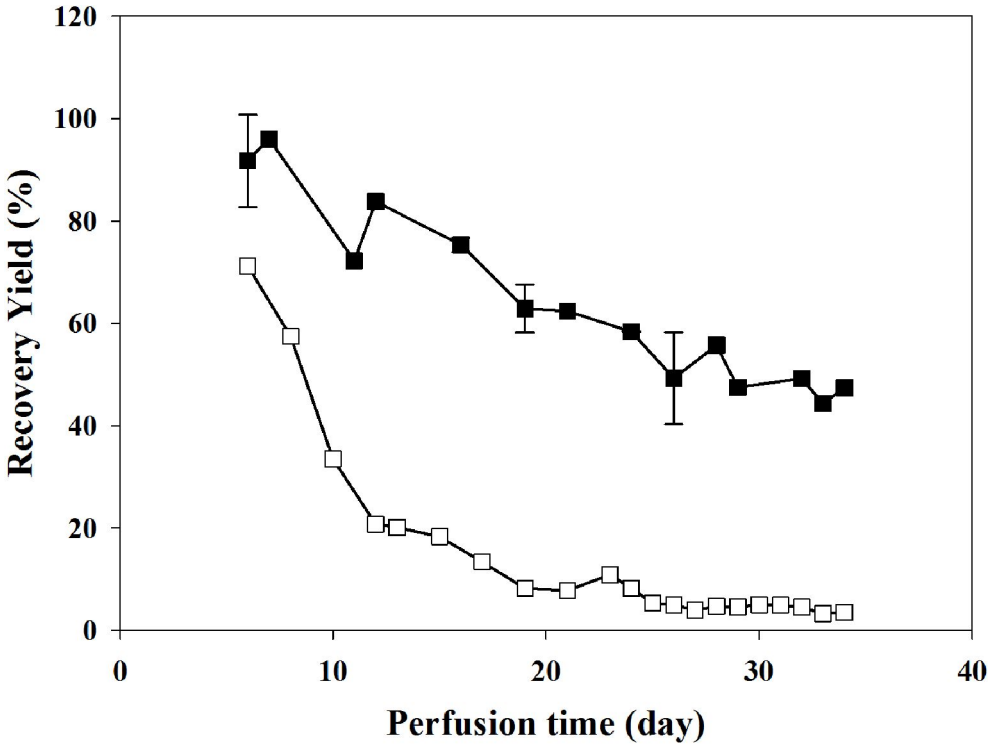


Fig. 5.2 FVIII:C recovery yield of ATF no. 1 run (open square) and ATF no. 2 run (closed square). Error bars represent standard deviations with n=2. The recovery yield was calculated by the ratio of FVIII:C in the harvest and in the bioreactor.

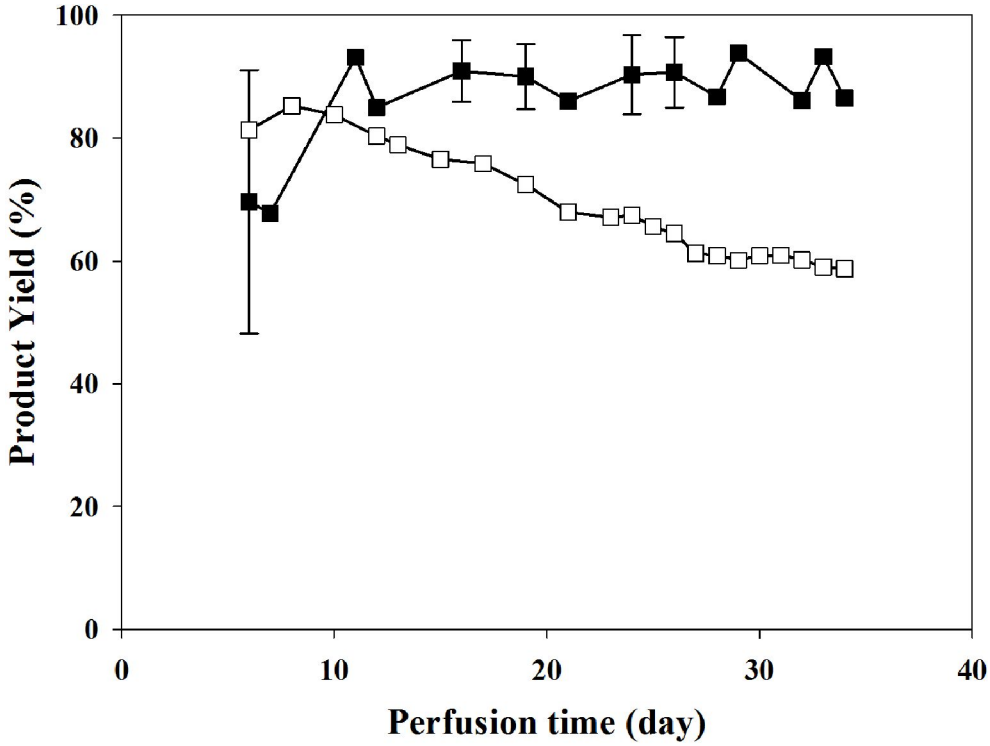


Fig. 5.3 FVIII:C product yield of ATF no. 1 run (open square) and ATF no. 2 run (closed square). Error bars represent standard deviations with n=2. The product yield was calculated by the ratio of the accumulated harvested FVIII:C and the total accumulated cellular production. The total accumulated cellular production = the accumulated harvested FVIII:C + the accumulated loss of FVIII:C by bleeding + the FVIII:C in bioreactor

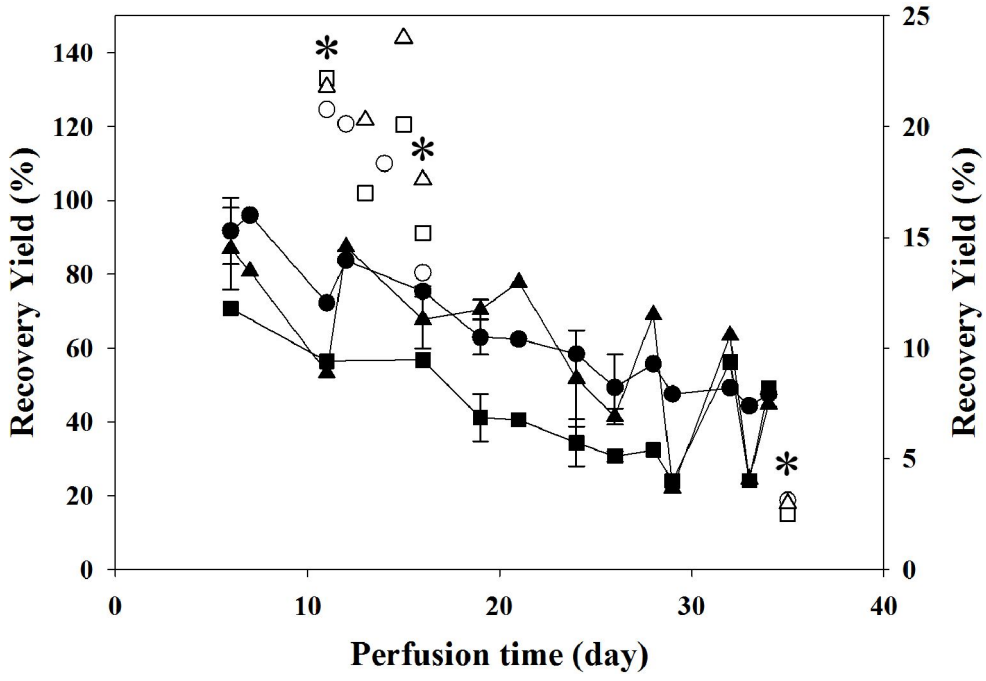


Fig. 5.4 Time profiles of FVIII:C recovery yield (circle), FVIII:Ag recovery yield (square) and vWF:Ag recovery yield (triangle). ATF no. 1 run: open symbol and right y-axis. ATF no. 2 run: closed symbol and left y-axis. Error bars represent standard deviations with n=2. *$P < 0.05$ (two-tailed Student's *t*-test).

Chapter VI

Conclusions and recommendations

In this thesis, it was reported for the first time that the FVIII yield in ATF perfusion culture is significantly low compared to spin-filter and Centritech Lab III perfusion culture. Since the reduction of target protein yield by fouling of the hollow fiber filter is an inherent problem of filtration based perfusion culture, the possibility of yield reduction due to fouling obviously exists even in ATF perfusion culture. Even after considering the possibility of reduction in protein retention caused by filter fouling in the bioreactor, the FVIII:C recovery yield was unusually low in this thesis.

The first study described in Chapter III aims to investigate the reasons for the unexpected low FVIII:C recovery yield in the ATF perfusion culture. It was assumed that three possible mechanisms could lead to a very low FVIII:C recovery yield in ATF perfusion culture. Firstly, the inactivation of secreted FVIII by temperature or various enzymes secreted by the CHO cells during storage of the harvest in the refrigerator until the replacement into a new harvest bag. Secondly, the reduction of the amount of FVIII:Ag itself by adsorption of FVIII molecule onto the harvest bag surface. Lastly, the reduction of the FVIII:Ag amount by molecular sieving through hollow fiber filter membrane.

To verify these assumptions, it was firstly examined the inactivation and surface adsorption of FVIII molecule during storage at 4^{o}C. The significance of TMP across the hollow fiber filter membrane was investigated. The results described in Chapter III successfully demonstrated that the low FVIII:C recovery yield in ATF perfusion culture was not due to the inactivation or surface adsorption of FVIII molecule, but due to the retention of the FVIII molecules by hollow fiber filtration. From the results of the controlled TMP experiments, the controlled TMP below 10psi resulted in remarkable enhancement of FVIII yield in hollow fiber filtration. From these observations, it was concluded that the TMP across hollow fiber filter is a key factor to increase the product yield of FVIII in ATF perfusion culture.

In Chapter IV, it was investigated how the TMP across hollow fiber filter can be increased. In a peristaltic pump harvest system, the increase of TMP was limited due to the continuous occlusion of harvest tube. Therefore, a modified harvest system was developed to overcome the limitation of a peristatic pump harvest system.

To maximize the effect of transmembrane on the FVIII yield, the modified harvest system consisting of check valve, pinch valve and timer was developed. The performance of the modified harvest system was compared to a peristaltic pump. It was demonstrated that the modified harvest system achieved the enhancement of the recovery and product yield of FVIII compared to the peristaltic pump system. The final recovery yield of FVIII:C was increased an approximately 13.6-fold and the product yield of FVIII:C was increased a 1.47-fold compared with a peristaltic pump. The core technology of the modified harvest system is the use of a check valve. A check valve is a one-way valve which allows the flow of fluid in only one direction. The check valve enables the permeate pressure to be minimized during the pressure cycle in ATF perfusion. This characteristic function of the check valve enabled to achieve the increased TMP and the enhancement of FVIII yield.

However, it is not clearly elucidated in this thesis that why the FVIII yield is decreased with the increased TMP over 10psi. It was inferred that the co-expressed vWF is another factor affecting the retention of FVIII:Ag because of the molecular size of the FVIII/vWF complex. Thus, subsequent studies investigating the effect of the co-expressed vWF on the FVIII yield as well as the mechanism by which the FVIII yield is unexpectedly decreased under the increased TMP at more than 10psi should be performed.

In addition, further studies are needed to develop a precise control method of TMP for the controlled TMP experiments. In this thesis, KrosFlo Research IIi system was used for the TMP control experiments. This system has a resolution problem of its

recording software used. The minimum data collection period of the recording software is 1 second. '1 second' does not seem to be enough to collect the data to show a precise TMP profile. Therefore, it was thought that if a higher-resolution recording software is used to collect data, a more precise TMP control can be obtained. Through the precise TMP control, it is possible to determine an optimal TMP for the maximum FVIII yield.

This thesis clearly showed that the modified harvest system developed in this thesis achieved the enhancement of FVIIII yield compared to the peristaltic pump harvest system. However, although the FVIII:C recovery yield was enhanced by using the modified harvest system as described in Chapter IV, the FVIII:C recovery yield was still lower than that of spin-filter and Centritech Lab III perfusion. The average recovery yield of FVIII:C in ATF perfusion with the modified harvest system was 64.6%. Whereas, the average recovery yield of FVIII:C was 92.8% in spin-filter perfusion and 81.7% in Centritech Lab III perfusion, respectively. These results indicate that ATF system has a limitation due to the molecular sieving of FVIII/vWF complex by hollow fiber filter. Thus, spin-filter or Centritech Lab III centrifuge system might be more efficient and suitable than ATF system in the production of FVIII co-expressed with vWF.

In conclusion, the modified harvest system of this thesis offers an efficient alternating method for harvesting in ATF perfusion culture. The system can be beneficial to other recombinant proteins production using ATF system as well. Another important point is that the system provides a promising tool to solve the fouling problem in the filtration-based perfusion system, especially in ATF perfusion culture.

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국문 요약

본 학위논문의 목적은 ATF 관류식 배양에서 혈액응고 제 8 인자 (Factor VIII)의 생산 수율을 향상시키기 위한 개선된 회수장치를 제공하는 것이다. 이를 위하여 체크밸브, 핀치밸브와 타이머로 구성된 개선된 회수장치를 개발함으로써 본 학위논문의 목적을 달성하였다.

본 학위논문은 세 개의 연구로 구성되었다. 첫 번째 연구에서는 ATF 관류식 배양에서 혈액응고 제 8 인자 활성 회수 수율이 낮은 이유에 대하여 고찰하였다. 폰 블리브란트 인자(vWF)와 동시에 발현되는 재조합 인간 혈액응고 제 8 인자의 생산을 위하여 관류식 배양에 널리 사용되는 ATF 시스템, spin-filter, Centritech Lab III centrifuge 등과 같은 세 가지의 세포체류장치(cell retention device)를 평가하였다. 그 결과, ATF 관류식 배양에서 다른 두 개의 세포체류장치에 비해 바이오리액터 안의 혈액응고 제 8 인자의 활성이 매우 높음을 발견하였다. 더욱이, 혈액응고 제 8 인자 활성 회수 수율이 ATF 관류식 배양에서 다른 두 개의 세포체류장치에 비해 예상외로 매우 낮음을 발견하였다.

첫 번째 연구결과를 통해 재조합 혈액응고 제 8 인자 분자의 회수용기표면으로의 흡착(surface adsorption)과 불활화(inactivation)는 ATF 관류식 배양에서의 낮은 수율에 대한 주요 원인이 아님을 확인하였다. 재조합 혈액응고 제 8 인자의 수율은 관류식 배양에 사용되는 중공사막(hollow fiber filter membrane)의 공극 크기(pore size)를 0.2μm 에서 0.5μm 로 증가시켰을 경우에도 향상되지 않았다. 또한, 중공사막에 의한 재조합 혈액응고 제 8 인자 분자의 잔류가 ATF 관류식 배양에서 낮은 혈액응고 제 8 인자 활성 수율의

주요 원인이었음을 알아내었다. 우리는 중공사막에 가해지는 막간압력(transmembrane pressure)를 조절함으로써 혈액응고 제 8 인자 수율이 크게 향상됨을 보여주었다. 이러한 연구결과를 종합하여보면 막간압력의 조절이 ATF 관류식 배양에서 혈액응고 제 8 인자의 수율 향상을 위한 효율적인 방법이 될 수 있음을 시사하고 있다.

두 번째 연구에서는 ATF 관류식 배양에서 혈액응고 제 8 인자 수율을 향상시키기 위한 개선된 회수장치의 개발에 대하여 기술하였다. 본 연구에서는 막간압력을 향상시킬 수 있는 개선된 회수장치를 개발함으로써 ATF 관류식 배양에서 낮은 혈액응고 제 8 인자 회수 수율을 향상시킬 수 있는 가능성을 제시하였다. 개선된 회수장치에서 주요한 혁신적인 개선점은 회수를 위한 연동펌프(peristaltic pump)의 사용을 배제할 수 있는 체크밸브와 핀치밸브의 사용이다. 혈액응고 제 8 인자가 포함되어있는 배양상등액 회수를 위한 관이 항상 막혀있는 연동펌프 회수장치에서는 ATF 관류식 배양에서 혈액응고 제 8 인자 수율 향상의 중요한 요인인 막간압력을 증가시키는 데 한계가 있다. 그러나, 개선된 회수장치에서는 연동펌프 대신 체크밸브를 사용함으로써 투과압력 (permeate pressure)을 44.8% 감소시킬 수 있었으며, 이로 인해 막간압력을 96%이상 향상시킬 수 있었다.

세 번째 연구에서는 개선된 회수장치의 혈액응고 제 8 인자와 폰 빌리브란트 인자를 동시에 발현하는 중국 햄스터 난소세포(Chinese hamster ovary(CHO) cell)의 관류식 배양에의 적용에 대하여 기술하였다. 개선된 회수장치는 연동펌프를 사용한 종래의 회수장치와 동등한 세포성장을 보여주었다. 관류속도(perfusion rate)도 개선된 회수장치를 이용하여 성공적으로 조절되었다. 또한, 개선된 회수장치는 연동펌프와 비교하였을 때 혈액응고 제 8

인자 활성의 최종 회수 수율을 약 13.6 배, 혈액응고 제 8 인자 활성의 생산 수율을 약 1.47 배 증가시켰다. 개선된 회수장치에 의한 혈액응고 제 8 인자 활성 수율의 향상은 혈액응고 제 8 인자 분자의 잔류량 (the retention of FVIII:Ag)의 감소에 기인하였다.

막간압력 측정의 결과로부터, 연동펌프 회수장치와 비교 시 잔류된 혈액응고 제 8 인자 분자의 감소는 증가된 막간압력에 기인하며, 이러한 막간압력의 증가는 체크밸브의 특징적인 기능에 의한 것임을 알게 되었다.

결론적으로 본 연구를 통해 개발된 개선된 회수장치를 이용하여 ATF 관류식 배양에서 재조합 혈액응고 제 8 인자 생산 수율을 향상시킬 수 있었다. 또한, 개선된 회수장치는 재조합 혈액응고 제 8 인자 뿐만 아니라 다른 재조합 단백질의 생산 수율을 향상시키는데 유용할 것으로 기대된다.

본 학위논문에서 얻은 또 다른 중요한 성과는 개선된 회수장치가 ATF 관류식 배양과 같은 여과기반 관류식 배양 (filtration-based perfusion) 시스템의 내재적인 문제인 분리막오손 (membrane fouling) 문제를 근본적으로 해결할 수 있는 유망한 방법을 제시하였다는 데 있다.

주요어: 혈액응고 제8인자, ATF perfusion, 개선된 회수장치, 막간압력, 핀치밸브, 체크밸브

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